The Role of the Oxygen-Sensing Prolyl-4-Hydroxylase Enzyme PHD2 in Tumor Formation

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1 Abbreviations

2-OG	2-oxoglutarate
AhR	aryl hydrocarbon receptor
ANG	angiogenin
ANGPT	angiopoietin
AREG	amphiregulin
ARNT	AhR nuclear translocator
BMDC	bone-marrow derived cells
BPD	bronchopulmonary dysplasia
CAC	circulating angiogenic cells
CAIX	carbonic anhydrase IX
CLI	critical limb ischemia
CO_2	carbon dioxide
CREB	cyclic AMP response element binding protein
DFO	desferrioxamine
DMOG	dimethyloxaloylglycine
ED	embryonic day
EGFR	epidermal growth factor receptor
EGL-9	egg-laying abnormal 9
EGLN	egl nine homolog
EPAS	endothelial PAS protein
EPO	erythropoietin
FGF	fibroblast growth factor
FIH	factor inhibiting HIF
GBM	glioblastoma multiforme
GLUT1	glucose transporter 1
GOF	gain of function
Hb	hemoglobin
Hct	hematocrit
HIF	hypoxia-inducible factor
HNSCC	head and neck squamous cell carcinoma
HPH	HIF prolyl-hydroxylase

HRE	hypoxia-responsive element
IL-8	interleukin-8
K_M	Michaelis constant
L-Mim	L-mimosine
MUC1	mucin1
NF-κB	nuclear factor κB
NO	nitric oxide
ODD	oxygen-dependent degradation
PAS	Per/ARNT/Sim
PDAC	pancreatic ductal adenocarcinoma
PDGF	platelet-derived growth factor
PHD	prolyl-hydroxylase domain enzyme
PlGF	placental growth factor
pO_2	oxygen partial pressure
pVHL	von Hippel-Lindau protein
RELA	v-rel reticuloendotheliosis viral oncogene homolog A
RNAi	RNA-interference
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SCF	stem cell factor
SDF1	stromal derived factor 1
shRNA	short-hairpin RNA
UTR	untranslated region
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WISP	wnt-inducible signaling protein
WRE	WT1 response element
Wt	wild type
WT1	Wilms tumor suppressor 1

2 Zusammenfassung

Die reduzierte Gewebe-Sauerstoffversorgung (Hypoxie) ist eine bekannte Eigenschaft der meisten soliden Tumoren. Tumor Hypoxie ist assoziiert mit erhöhter Bösartigkeit, erhöhter Zellmigration und Invasion, verstärkter Angiogenese sowie mit einer höheren Resistenz gegen Chemo- und Radiotherapie. Die molekularen Änderungen die zu einer zellulären Hypoxieadaptation führen, werden grösstenteils durch heterodimerische Hypoxie-induzierbare Faktoren (HIFs) gesteuert. Es wurde gezeigt, dass die HIFs und die meisten ihrer Zielgene eine wichtige Rolle in der Krebsentstehung spielen. Degradation der α Untereinheit des HIF Transkriptionsfaktors wird durch molekularen Sauerstoff messende Eiweisse reguliert, die so genannten Prolyl-4-Hydroxylase Domäne Proteine (PHDs). Die drei bekannten PHD Isoformen (PHD1, 2, 3) hydroxylieren die α Untereinheit in Anwesenheit von molekularem Sauerstoff (O_2). Es wurde gezeigt, dass die hydroxylierte HIF-α Untereinheit an einen E3-Ubiquitin-Ligase Komplex bindet, der als Erkennungsuntereinheit das von-Hippel-Lindau Tumorsuppressorprotein (pVHL) beinhaltet. Die Interaktion mit pVHL führt zur proteasomalen Degradation. Unter hypoxischen Bedingungen findet wegen des Mangels an Sauerstoff keine Hydroxylierung statt. Als Folge davon wird die HIF-α Untereinheit stabilisiert und migriert in den Zellkern, wo sie mit der stabil exprimierten HIF-β Untereinheit heterodimerisiert. Das Binden des HIF-Heterodimers an spezifische DNS Sequenzen, die so genannten Hypoxie „responsive“ Elemente (HREs), führt zur Transaktivierung von Zielgenen.

Um den Beitrag von PHD2 in der Krebsentstehung und Entwicklung zu untersuchen, haben wir PHD2 short-hairpin (sh)RNS Klone in MCF-7 Brustkrebszellen generiert. Die Resultate von *in vivo* Xenograft Versuchen in Nacktmäusen zeigen, dass PHD2 Herunterregulierung in MCF-7 zu schneller wachsenden Tumoren führt. Die Rekonstitution von PHD2 in den knock-down Klonen führte zu einer deutlichen Abnahme des Tumorwachstums. Die *in vitro* Zellproliferation wurde nicht von der PHD2 Menge beeinflusst. Gewebe Mikroarray Analysen der PHD2 Expression in 281 klinischen Brustkrebsproben zeigten eine signifikant reduzierte Überlebenszeit, über eine Zeitspanne von zehn Jahren, bei Patientinnen mit niedrigen PHD2 Protein in den Tumoren.

Die Analyse von PECAM-1 Färbungen auf gefrorenen Tumorschnitten zeigte, dass sowohl die Blutgefässdichte, als auch der Blutgefässdurchmesser und die Blutgefässoberfläche in den shPHD2 Tumoren erhöht waren. Um diesen angiogenetischen Phänotyp weiter zu untersuchen und um Unterschiede in der Sekretion von angiogenetischen Faktoren festzustellen, wurde Zellkulturmediumüberstand von MCF-7 Wildtyp Zellen, shPHD2 und von rekonstituierten PHD2 Klonen in einem humanen Angiogenesearray getestet. Der vaskuläre Endothel Wachstumsfaktor A (VEGF-A) war in den PHD2 hinunterregulierten Zellen im Vergleich zu den Wildtyp und rekonstituierten Klonen induziert. Dies deutet auf eine Aktivierung des klassischen HIF-Signalwegs hin. Ebenso waren auch die Mengen an Interleukin-8 (IL-8) und Amphiregulin (AREG) durch die PHD2 Menge beeinflusst, mit einer höheren Expression bei Abwesenheit von PHD2 und einer tieferen Expression nach PHD2 Rekonstitution.

Sowohl AREG mRNS Mengen als auch AREG Promoteraktivität waren in Abwesenheit von PHD2 erhöht und wieder tiefer nach PHD2 Rekonstitution. Wir konnten weiter zeigen, dass der PHD2 Effekt von der PHD2-Hydroxylierungsaktivität unabhängig ist und dass AREG mRNS Mengen nicht durch Hypoxie beeinflusst werden. Funktionell konnten wir zeigen, dass AREG die *in vitro* Gefässbildung positiv beeinflusst. Die AREG mRNS Menge sowie die Promoteraktivität werden durch HIF-2α aber nicht durch HIF-1α Expression beeinflusst.

Aus unseren Versuche entnehmen wir, dass PHD2 ein potentieller Tumorsuppressor sein könnte und dass die AREG-Regulation durch PHD2 und HIF-2α wichtig für das Brustkrebswachstum sein könnte. Weiter haben wir uns auf AREG und wnt-induzierbares Signalprotein (WISP) 2 konzentriert, beides spezifische HIF-2α Zielgene. Die Überlebenszeit von Brustkrebspatientinnen war deutlich verbessert bei gleichzeitiger AREG und WISP2 Expression im Tumor. Im Brustkrebs konnte eine starke Korrelation zwischen AREG, WISP2 und HIF-2α festgestellt werden. Eine detaillierte AREG-Promoter Analyse zeigte, dass mehrere Teile für den HIF-2α vermittelten Effekt nötig sind. Funktionell wurde in shHIF-2α Zellen eine reduzierte Phosphorylierung der Mitglieder der epidermalen Wachstumsfaktorrezeptor Familie (EGFR) beobachtet, im Vergleich zu Wildtyp und shHIF-1α Zellen. Die Phosphorylierung konnte durch Zugabe von exogenem AREG wieder rekonstituiert werden.

Zusammenfassend konnten wir zeigen, dass PHD2 und HIF-2α durch AREG-abhängige Angiogeneseinduktion und EGFR Aktivierung zum Brustkrebsmetabolismus beitragen können. Die Frage, ob PHD2 und HIF-2α im gleichen Signalweg agieren oder unterschiedliche Zielgene haben, lässt sich aber noch nicht endgültig beantworten.

3 Summary

Reduced tissue oxygenation (hypoxia), a characteristic feature of most solid tumors, is associated with increased malignancy, enhanced cell migration and invasion, increased angiogenesis as well as resistance to chemo- and radiotherapy. Cellular adaptation to hypoxia is mainly triggered by heterodimeric hypoxia-inducible factors (HIFs). HIFs and many of their target genes were shown to be implicated in the malignant progression of cancer. Degradation of the α subunit of the HIF transcription factor is regulated by molecular oxygen-sensing proteins, named prolyl-4-hydroxylase domain proteins (PHDs). The three known PHD isoforms (PHD1, 2 and 3) hydroxylate the HIF-α subunit in the presence of molecular oxygen (O_2). The hydroxylated HIF-α subunit was shown to bind an E3 ubiquitin ligase complex, containing as a recognition subunit the von Hippel-Lindau tumor suppressor protein (pVHL). Interaction with pVHL leads to proteasomal degradation. Under hypoxic conditions, lack of oxygen causes hydroxylation to cease. Therefore, the HIF-α subunit becomes stabilized and translocates to the nucleus where it heterodimerizes with the constitutively expressed β subunit. Binding of the HIF-heterodimer to specific DNA sequences, named hypoxia-responsive elements (HREs), triggers the transactivation of target genes.

To study the role of PHD2 in tumor formation and progression, we generated stable PHD2 short-hairpin (sh)RNA knock-down clones in MCF-7 breast cancer cells. Results obtained from *in vivo* xenografts experiments in nude mice suggest that PHD2 down-regulation in MCF-7 cells leads to faster growing tumors. Reconstitution of PHD2 in the MCF-7 knock-down clones abolished this effect. Importantly, *in vitro* proliferation was not affected by PHD2 levels. Conversely, tissue microarray analysis of PHD2 expression in 281 clinical samples of breast cancers showed significantly reduced survival over a period of ten years, in patients having low level PHD2 tumors, suggesting that PHD2 functions as tumor suppressor.

Analysis of PECAM-1 stainings in tumor sections revealed higher vascularization in tumors derived from shPHD2 cells. To further investigate the observed angiogenic phenotype and to determine differences in the secretion of angiogenic factors, conditioned media from parental MCF-7 cells, shPHD2 clones and reconstituted PHD2 clones were tested in a human angiogenesis array. Vascular endothelial

growth factor A (VEGF-A), interleukin-8 (IL-8) and amphiregulin (AREG) protein levels were found to be affected by PHD2 levels, with higher expression in the absence of PHD2 and decreased expression after PHD2 reconstitution.

AREG mRNA levels as well as AREG promoter activity were found to be increased in the absence of PHD2 and decreased again after PHD2 reconstitution. In addition, the PHD2 effect was shown to be independent of PHD2 hydroxylase activity and AREG mRNA levels were not altered by hypoxia. Functionally, we could show that AREG positively influences *in vitro* tube formation. AREG mRNA levels as well as promoter activity were found to depend on HIF-2α but not HIF-1α expression.

From our experiments we conclude that PHD2 is a potential tumor suppressor in breast cancer and that AREG regulation by PHD2 as well as HIF-2α might contribute to breast tumorigenesis. Furthermore, we concentrated on AREG and wnt-inducible signaling protein (WISP) 2 as specific HIF-2α target genes. Survival of breast cancer patients was significantly improved by the concomitant expression of AREG and WISP2. Moreover a strong correlation was found between AREG, WISP2 and HIF-2α in breast tumors. Analysis of the AREG promoter led to the conclusion that multiple sites are necessary to mediate the HIF-2α effect on AREG transcription. Functionally, a decreased phosphorylation of epidermal growth factor receptor (EGFR) family members was observed in HIF-2α deficient cells compared to HIF-1α deficient cells and parental MCF-7 cells. Phosphorylation of EGFR family receptors in HIF-2α cells was rescued by addition of exogenous AREG.

Summarizing, we conclude that PHD2 and HIF-2α contribute to breast cancer metabolism by affecting angiogenesis as well as EGFR signaling in an AREG-dependent manner. However, if PHD2 and HIF-2α act on the same pathway or have different targets is not yet clear.

4 Introduction

4.1 Hypoxia and hypoxia tolerance in mammals

4.1.1 Hypoxic environments

Hypoxia is defined as a reduced oxygen partial pressure (pO_2) that causes a reduction in oxygen availability to body tissues. In general, mammals experience hypoxia during diving, hibernation or burrowing. For human beings exposure to hypoxia is usually correlated to a stay in the mountains at high altitudes. Additionally, hypoxia plays an important role during development. Responses to hypoxia can be acute, meaning that they will take place within seconds to minutes or chronic, meaning that they will last for hours or days. Many physiological adaptations to hypoxia, like differential vasoconstriction, bradycardia, and hypometabolism are shared among all mammals. Hypoxia tolerance is achieved through a complex reorganization, coordination and partial shut-down process of different organs[1].

4.1.2 Adaptation to acute hypoxia

In diving mammals, ventilation stops immediately causing a continuous decrease in arterial oxygen and a parallel increase in arterial carbon dioxide (CO_2)[2]. All diving animals have an increased oxygen storing capacity in the blood, muscles and lungs. In the blood, this is achieved with a high hematocrit (Hct) and high haemoglobin (Hb) concentration. The mass of the red blood cells was found to positively correlate with diving capacity[3]. The high oxygen affinity of muscular myoglobin (Mb), present at high concentrations, is a useful oxygen store and also provides antioxidant effects[4, 5]. Common features in diving animals are high capillary densities in the brain tissue as well as a high tidal volume exchange during the brief periods of ventilation that enable to rapidly discard CO_2 and load oxygen[6-8]. Since increased oxygen storage alone is not sufficient to survive acute hypoxia, animals also have to reduce their oxygen requirements, a condition called hypometabolism[2, 3]. Importantly,

neuroprotection and functional integrity need to be guaranteed also under hypometabolic conditions.

4.1.3 Adaptation to chronic hypoxia

Adaptation to chronic hypoxia is different from adaptation to acute hypoxia. Increased oxygen storage and hypometabolism are not long-term options. The strategy to survive chronic hypoxia involves improved oxygen affinity and increased oxygen supply. Acclimatization to high altitudes is characterized by carotid body-mediated hyperventilation to enhance oxygen uptake[9, 10], increased cardiac output to deliver oxygen to tissues, and erythropoietin (EPO)-induced increase in red blood cells (RBC) and subsequent Hb amount[11, 12]. Interestingly, these short-term adaptations to chronic hypoxia are very different from the strategies applied by mammals, which permanently live at high altitudes. In fact, to couple high oxygen carrying capacity with normal blood viscosity, these adapted animals show low Hb and Hct levels, but a high Hb-oxygen affinity[13-16].

4.1.4 Pathological hypoxia

From a medical point of view, hypoxia is defined as an abnormal condition in which the whole body or single body tissues are deprived of an adequate oxygen supply. Hypoxia is a feature of many common pathological conditions such as stroke, ischemia, inflammation or cancer. The beginning of the history of research on tumor hypoxia can be traced back to the early 20th century when Otto Warburg demonstrated that tumor cells favor glycolysis independent of oxygenation levels[17]. In the second half of the past century, hypoxic cells could not yet be visualized, but their existence was highly postulated. Observations of necrotic areas in absence of capillaries in histological sections of human tumors as well as irradiation experiments in mice exposed to different oxygen pressures gave hints for the existence of hypoxic cells but direct evidence was missing[18, 19]. Studies in the 1980s and 1990s using ^{31}P-nuclear magnetic resonance (NMR) to monitor cell metabolism, misonidazole[20, 21] to selectively bind hypoxic cells, electrode measurements and cryospectrophotometric measurements of oxyhemoglobin[22], confirmed the lower

oxygen tension in cancer tissues. The main focus of researchers in the 20th century was the role of hypoxia in responses to cancer treatments. Since 1909 it was known that cells under hypoxic conditions are less sensitive to radiation, but the changes were attributed to differences in blood flow[23]. Hypoxic radioresistance was demonstrated by Gray and colleagues in the 1950s and later confirmed in many other studies[24]. Similarly, common chemotherapeutic agents were shown to be less effective against hypoxic cells[25, 26]. In solid tumours, hypoxic conditions are not only associated with resistance to therapies, but also with increased malignancy, enhanced invasiveness and angiogenesis.

4.2 Hypoxia-inducible factor (HIF)

4.2.1 The transcription factor HIF

The hypoxia-inducible factor 1 was first described by Semenza and Wang in 1992[27], who observed a factor able to bind the 3'-hypoxia responsive element (HRE) of the EPO gene only under hypoxic conditions. Later HIF-1 was shown to be part of a wide-spread oxygen-sensing and signal-transduction mechanism[28, 29]. Purification of HIF-1 revealed that it is a heterodimer consisting of an α and a β subunit[30]. The β subunit was found to be identical to the previously identified dimerization partner of the dioxin receptor/aryl hydrocarbon receptor (AhR), called AhR nuclear translocator (ARNT). Sequencing of the novel HIF-1α subunit led to the identification of a basic helix-loop-helix (bHLH) and of a Per/ARNT/Sim (PAS) domain[12]. Three different α-subunits (HIF-1α, HIF-2α and HIF-3α) and one β-subunit (HIF-1β) are known so far. The HIF-2α isoform is also called endothelial PAS protein 1 (EPAS1). While HIF-1α is ubiquitously expressed in mice, HIF-2α expression was originally thought to be restricted to embryonic and adult endothelium[31]. The full length splice variant of HIF-3α is mainly found in the kidney[32].

4.2.2 Oxygen-dependent regulation of HIF-α subunits

HIF-α protein levels, but not HIF-α mRNA levels are strongly regulated in an oxygen-dependent manner. Under normoxic conditions, specific proline residues located in the oxygen-dependent degradation (ODD) domain are hydroxylated by the HIF prolyl-4-hydroxylases (PHDs). This hydroxylation is a prerequisite for binding of the von Hippel-Lindau (pVHL) tumor suppressor protein and subsequent ubiquitin-dependent degradation of the HIF-α subunits by the 26S proteasome[33-36]. Normoxic HIF-a stabilization due to loss of pVHL was documented in vascular tumors like renal clear cell carcinoma, hemangioblastoma and pheocromocytoma[35].
Because of decreased PHDs activity at low pO_2, HIF-α subunits become stabilized and translocate to nucleus, where they heterodimerize with the constitutively expressed HIF-1β subunit. The HIF heterodimer binds to hypoxia responsive elements (HRE) and triggers the expression of HIF target genes[37]. Additionally, hydroxylation of a specific asparagine residue in the C-terminal transactivation domain of the HIF-α subunit by the factor inhibiting HIF (FIH), interferes with the binding of the HIF-α subunit to p300/CBP preventing transcriptional activation of the HIF complex[38, 39].

4.2.3 Targeted knock-out of HIF subunits

While the genetic ablation of the HIF-3α subunit in mice has not yet been reported, the targeted knock-out of HIF-1α and HIF-2α led to partially different and non redundant phenotypes. Severe defects in neuronal tube, vascular and pharyngeal structures formation as well as in cardiovascular development leading to death at embryonic day (ED) 10.5 were observed after loss of HIF-1α[40, 41]. The developmental defects occurring after a HIF-2α targeted knock-out seemed to be restricted to the cardiovascular system. In addition, different phenotypes were observed depending on the mouse strain used for the experiment. In a C57BL/6 background, loss of HIF-2α led to embryonic death following defects in cardiac and circulatory functions due to reduced catecholamine production[42]. ICR/129 sv mice lacking HIF-2α presented lethal defects during remodelling of the primary vascular network[43].

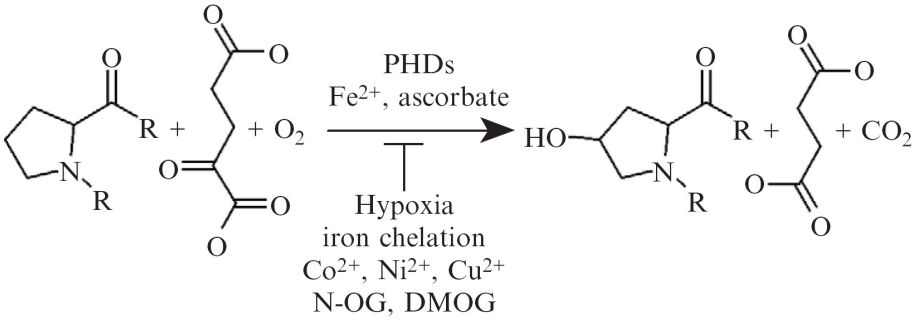
4.3 HIF prolyl-4-hydroxylases (PHDs)

4.3.1 The prolyl-4-hydroxylases

The first HIF prolyl-4-hydroxylase was characterized in *C. elegans* in 2001[44] and was named egg-laying abnormal 9 (EGL-9). Three ortholog isoforms could be identified in humans and were named PHD1 (alternatively termed egl nine homolog [EGLN] 2, or HIF prolyl-hydroxylase [HPH] 3), PHD2 (EGLN1, HPH2), and PHD3 (EGLN3, HPH1). Since these proteins were shown to be able to modulate HIF-α stability in an oxygen-dependent manner, they are considered to be the cellular oxygen sensors[45, 46]. Alternative splicing variants have been described for PHD2[47] and PHD3[48] and a shorter PHD1 isoform results from alternative translational initiation[49]. A fourth PHD-related protein (PH-4), whose functional relevance is not yet understood, has been identified[50]. Like the collagen prolyl-hydroxylases, also the HIF prolyl-hydroxylases belong to the iron and 2-oxoglutarate (2-OG)-dependent family of dioxygenases.

4.3.2 The enzymatic reaction

Molecular oxygen, Fe^{2+} as well as 2-OG are essential for the PHD catalyzed enzymatic reaction to occur. During the enzymatic reaction one oxygen atom is transferred to position 4 of a prolyl residue of the substrate forming hydroxyproline. The second oxygen atom is utilized for the decarboxylation reaction which will convert 2-OG to succinate with formation of CO_2 [Figure 1][51].



proline 2-oxoglutarate 4-hydroxyproline succinate

Figure 1. The enzymatic reaction catalyzed by HIF prolyl-4-hydroxylases[51]

Furthermore, ascorbate is thought to be needed *in vitro* for functional catalysis. This can be explained with the evidence that ascorbate protects the ferrous iron (Fe^{2+}) in the active site from oxidation to ferric iron (Fe^{3+})[52]. The PHDs hydroxylate two specific proline residues located in the ODD domain of the HIF-α subunit, namely P402 and P564. *In vitro*, P564 can be hydroxylated by all three isoforms, whereas P402 was found to be hydroxylated only by PHD1 and PHD2[44]. However, overexpression experiments with all three isoforms in cellular systems showed that all of them are active on both proline residues[53]. Under normoxic conditions hydroxylation of P564 is necessary for hydroxylation of P402 to occur. Under hypoxic conditions, hydroxylation of P402 starts to be inhibited prior to P564 hydroxylation. This inhibition of P402 hydroxylation was shown to be already sufficient for HIF-α stabilization at low pO_2 although P564 hydroxylation was still taking place[53]. In cells, PHDs contribute differentially to HIF-α hydroxylation based on their expression levels[54]. The hydroxylation targets proline residues in a conserved LXXLAP motif (X means any random amino acid) of the HIF-α subunit. Only the proline in this motif is absolutely necessary for recognition[55-57].

4.3.3 Tissue distribution and targeted knock-out of PHDs

The levels of PHD expression in the different organs and tissues is very variable. While PHD2 seems to be expressed almost everywhere, peaks of PHD3 expression are found in the heart and highest PHD1 expression was detected in testis[50, 58]. Experiments carried out with fluorescent-fusion or tagged proteins at the subcellular level, showed that PHD1 was exclusively detected in the nucleus and PHD2 in the cytosol. PHD3 was almost equally distributed between the two compartments[55, 59]. However, in rat hepatocytes endogenous PHD2 and PHD3 were both described in the cytoplasm as well as in the nucleus[60].

PHD1 and PHD3 knock-out in mice do not affect embryonic survival, whereas a targeted disruption of the PHD2 locus causes the death of the embryo, due to severe defects in the heart. Heart defects were not accompanied by high HIF–α protein levels, suggesting either a compensatory role for the remaining isoforms or additional HIF-independent functions of PHD2[61].

4.3.4 PHD - HIF feedback loop

It was shown that PHD2 and PHD3 but not PHD1 are direct HIF targets. As a consequence, their mRNA levels were shown to be induced under reduced pO_2[62]. Proline hydroxylation is an irreversible process and the enhanced hydroxylation that takes place following PHD accumulation under hypoxic conditions, accelerates the rate of HIF-α degradation during reoxygenation[63, 64]. This feedback loop is also important under conditions of prolonged hypoxia, where increased PHD levels can partially compensate for the low pO_2 and therefore limit the hypoxic response elicited by HIF protein accumulation[65].

4.3.5 Factors influencing PHD activity

PHD activity is influenced by various factors. The most important among them is oxygen, whose presence is essential for the hydroxylation reaction. Michaelis-constant (K_M) values for all three PHDs were determined by an *in vitro* hydroxylation-coupled decarboxylation assay and range between 230-250 μM under normoxic conditions, meaning that changes in pO_2 can easily affect HIF-α prolyl-hydroxylation[47]. These K_M values are clearly higher than tissue pO_2 providing the basis for the PHD catalyzed enzymatic reactions[66].

The prolyl-hydroxylase reaction requires 2-OG, an intermediate of the citric acid cycle. However, other metabolites, namely oxaloacetate or pyruvate were shown to inhibit the hydroxylation reaction and therefore to promote HIF-α accumulation, by occupying the 2-OG binding site [67, 68].

Like all members of the Fe(II)- and 2-oxoglutarate-dependent dioxygenase family, PHDs bind iron with high affinity. In addition to Fe^{2+} also other divalent cations, namely Cd^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+} were shown to have an impact on the HIF system[69, 70]. Cu^{2+} was shown to interact with the active site of the enzyme displacing iron and therefore stabilizing HIF-α under normoxic conditions[71]. Experiments carried out with Ca^{2+} chelators showed that calcium interferes with PHD2 activity, thereby inhibiting its effects[72].

Another molecule found to mediate HIF-α stabilization through its inhibitory effects on PHD activity is nitric oxide (NO), which is derived from different NO donors or produced by iNOS. The inhibitory effect might be due to formation of nitrosothiols that compete with oxygen or might be due to iron oxidation in the catalytic centre[67, 73, 74]. Interestingly, several studies revealed that NO can decrease hypoxia induced HIF-1α levels. This bimodal response to NO treatment can be explained by the fact that during early phases, in the presence of NO, PHD2 is inhibited and HIF-1α subsequently stabilized, whereas later when PHD2 protein levels are increased and concomitantly NO is no longer present, HIF-1α levels are again decreased[75, 76].
Reactive oxygen species (ROS) production is increased under hypoxic conditions because of inhibition of the respiratory chain. The evidence that antioxidants influence the oxygen signalling pathway suggest that ROS might play an important role in influencing the HIF system[77]. Experiments carried out with ascorbate as an antioxidant have shown that it can prevent iron oxidation in the catalytic centre of PHDs and enhance *in vitro* hydroxylation of a HIF-α ODD fusion protein construct[67, 78]. Two other proteins shown to play a role in the HIF system in association with ROS, are mucin 1 (MUC1) and Jun D. MUC1 attenuates HIF-transcriptional activity by inducing PHD3 and suppressing ROS accumulation[79]. The transcription factor Jun D, involved in oxidative stress defense, inhibits prolyl hydroxylation by increasing hydrogen peroxide (H_2O_2) metabolism, and consequently iron oxidation[80].

4.4 Prolyl-4-hydroxylase inhibitors

The prolyl-hydroxylases are increasingly considered as attractive drug targets for the treatment of different pathologies characterized by ischemia, inflammation or kidney failure. Based on studies showing that PHD2 can be a key regulator of HIF-driven angiogenesis[81], prolyl-hydroxylase inhibitors became of interest as potential therapeutic angiogenesis inducers.

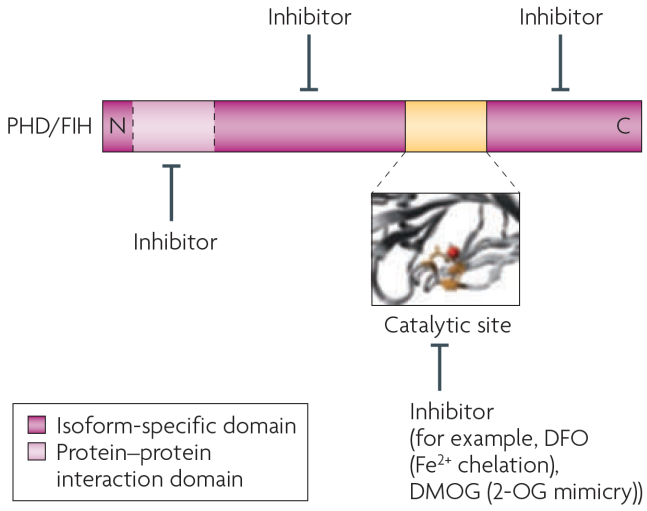


Figure 2. Targets of PHD-inhibitors[83]

In this context, it should not be forgotten that loss of PHD2 leads to death during mice development meaning that PHD inhibition has to be carefully modulated[82]. Newly designed inhibitors were shown to interfere with various domains of the protein such as its protein-protein interaction domain, the catalytic domain or, to increase specificity, with isoform-specific domains [Figure 2][83].

4.4.1 Non-selective PHD inhibitors

The catalytic site of the prolyl-hydroxylases can be inhibited either by compounds that structurally mimic 2-OG and therefore block the entry of the substrate or by iron-chelating compounds. Isoform specificity is not achieved with these compounds since 2-OG docking to the active site relies on amino-acids, which are highly conserved among the prolyl-hydroxylases (see Table 1 in reference[83]). Iron-chelation will also interfere with other enzymes having iron in their catalytic centre leading to undesired side-effects. Other metal ions such as Co^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} are not able to support PHDs enzymatic activity and can therefore also function as inhibitors[84].

To overcome the disadvantage of missing selective isoform inhibition, new compounds based on aromatic heterocycles related to pyridine derivatives, like 8-hydroxyquinolines are being developed[85]. Co-crystallization experiments with PHD2 showed promising interaction between these compounds and the enzyme[86].

4.4.2 Alternative approaches of PHD inhibition

Apart from the catalytic site, which is strongly conserved among PHDs, other domains like the amino- or the carboxy- terminal end, which show higher variability could be targeted. In fact two β-strands determine selectivity of PHDs for the N- or C- ODD. Since PHD3 seems to preferentially hydroxylate the CODD, while PHD2 hydroxylates both, inhibitors targeting this domain could enhance isoform-selectivity[87]. In addition, novel inhibitors could also target specific interaction partners like the isomerase FKBP38 that was shown to decrease PHD2 stability[88] or mitogen-activated protein kinase organizer 1 (MORG1) that was described as a scaffold protein for PHD3[89].

4.5 Angiogenesis

While vasculogenesis is defined as the *de novo* formation of blood vessels from endothelial cell precursors called angioblasts, angiogenesis describes the process of growth of new blood vessel from existing ones. Arteriogenesis is a remodelling of blood vessels, which results in an increased luminal diameter and a subsequent increase in blood flow. Angiogenesis is an essential physiological process especially during organ development. In adults, angiogenesis is mainly restricted to the female reproductive system, to organs undergoing physiological growth and to wound healing. Imbalanced angiogenesis is a common feature of numerous inflammatory, malignant, ischemic, infectious and immune disorders. Nowadays anti-angiogenic compounds are used to treat tumors and blindness.

4.5.1 Angiogenic switch

In addition to all genetic and epigenetic changes leading to tumor progression, another step is required for cancer cells to proliferate and expand: the induction of a tumor vasculature termed the angiogenic switch [Figure 3][90]. In fact, the generation of a functional tumor blood supply is a critical step in tumor progression. Induction of

the angiogenic switch depends on the balance between pro-angiogenic stimuli and angiogenesis inhibitory effects and can occur at different stages of tumor progression[91].

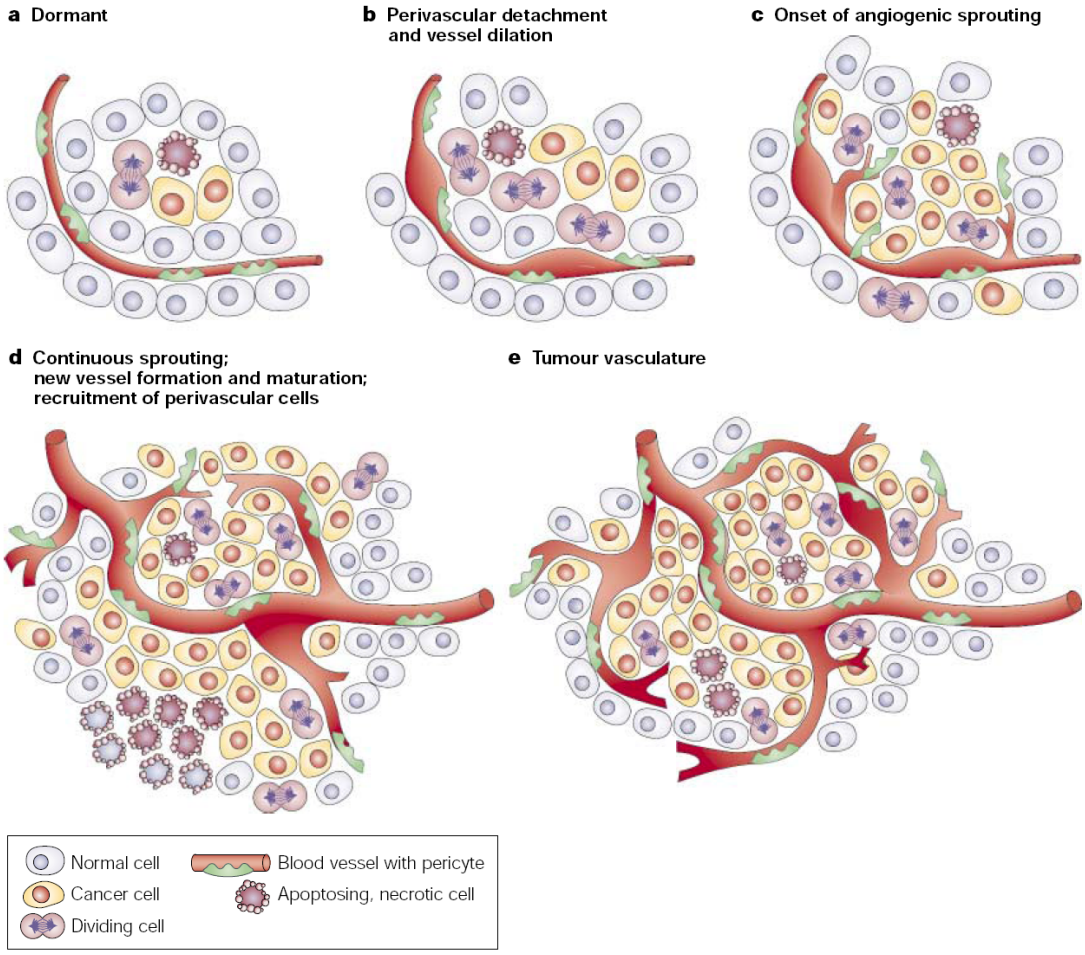


Figure 3. The angiogenic switch[90]

4.5.2 Tumor angiogenesis

In contrast to normal physiological angiogenesis where blood vessels rapidly mature and become stable, tumor angiogenesis leads to vessels which fail to completely develop and become quiescent. Tumor vessels are irregularly shaped, dilated and present dead ends lacking a typical organization in venules, arterioles and capillaries. These chaotic features make the tumor vascular network leaky and hemorrhagic. In addition, perivascular cells, which usually line the endothelium are

loosely associated and less abundant in tumor vessels[92, 93]. Even cancer cells were found to be intercalated in the vessel walls[94, 95]. In addition, some tumors mainly rely on vasculogenesis through recruitment of bone marrow-derived endothelial precursor cells rather than classical angiogenesis[96].

4.5.2.1 Formation of tumor blood vessels

Although the vasculogenesis process can be adapted for the use by tumors, with endothelial precursor cells mobilized from the bone marrow and transported to the walls of growing blood vessels, most tumor neovascularization occurs via angiogenesis[97]. In the first stage of angiogenesis, existing capillaries dilate and increase their permeability in response to VEGF, which was shown to be sufficient for the induction of angiogenesis in a quiescent vasculature[98]. Increased permeability allows extravasation of plasma proteins that form a provisory matrix on which endothelial cells can migrate[98]. While targeted degradation of the matrix and of the basement membrane allows endothelial cells to migrate, their continuous proliferation leads to enlargement of the blood vessel diameter. The decreased vessel association with pericytes in combination with the high production of VEGFA might in part explain the abnormal vessel diameters and the sensitivity to anti-VEGFA therapy[92, 99]. Recent studies have shown that the role of VEGFA could also be taken over by other angiogenic factors like angiogenin (ANG) [100, 101], platelet-derived growth factor (PlGF)[102], fibroblast growth factor (FGF) or other members of the VEGF family[103]. New blood vessels develop at the rim between hypoxic and necrotic tumor areas, where the VEGF levels are highest. Although cancer cells have a metabolism adapted to hypoxic conditions with an increased glycolysis to maintain ATP production[104], oxygen supply by blood vessels remains fundamental.

4.5.2.2 Tumor progression through angiogenesis

Various studies with mouse as well as human tumor models showed that angiogenesis can be switched on at different stages of tumor progression depending on the cancer type and on the surrounding microenvironment. The angiogenic switch was thought to separate two distinct phases in tumor progression; a first avascular phase characterized by small dormant lesions and a second one in which a small fraction of cancer cells will be able to proliferate, because of the growth of blood

vessels. This same principle also applies to metastasis[105]. The need of blood supply for tumor progression was proven in several studies. RipTag mice which develop pancreatic islet carcinomas as well as K14-HPv16 mice, used as a model of squamous cell carcinoma of skin and cervix, need neovascularization as a prerequisite for tumor progression[106-110]. VEGF was shown to be essential for the angiogenic switch in the pancreatic islet tumor model[107]. Mammary carcinoma prone mice (MMTV-neu) lacking the angiogenesis inhibitor thrombospondin-1 (TSP1) were found to give rise to bigger, more vascularized tumors[111]. Importantly, similar results were also observed in patients suffering from breast, prostate, and cervix carcinomas[91]. No human tumor bypassing the premalignant stage has been described to date.

4.5.2.3 The special case of astrocytomas

Although in most tumor types neoangiogenesis is an essential condition for further proliferation and growth, a type of brain tumors, namely astrocytomas, do not initiate angiogenesis during their growth. Blood supply is guaranteed by the alignment along existing brain blood vessels. These non angiogenic tumors do not possess a core and the tumor mass proliferates along the existing vessels[112]. Other very aggressive tumors, like chondrosarcomas, also present a low vessel density[113].

4.5.3 Anti-angiogenic therapy

Several angiogenesis inhibitors have been developed to block tumor angiogenesis and therefore slow-down tumor progression. The strategy behind this approach is to target cells that support cancer progression, like the endothelial cells forming the tumoral vasculature rather than cancer cells themselves.

4.5.3.1 Classes of angiogenesis inhibitors

Angiogenesis inhibitors can be subdivided into direct and indirect inhibitors [Figure 4][105]. Direct inhibitors target genetically stable endothelial cells preventing them from proliferating or migrating in response to pro-angiogenic stimuli. Indirect inhibitors block tumor-derived proteins, which induce angiogenesis or block their receptors on

endothelial cells. Since direct angiogenesis inhibitors are developed to target genetically stable endothelial cells, they are less prone to induce drug resistance[114]. Many of the targets of indirect inhibitors are oncogene products involved in the angiogenic switch[115-117]. In fact, activating mutations in oncogenes have been shown to lead to an induction of pro-angiogenic molecules and to a simultaneous down-regulation of angiogenesis inhibitors[115]. Certainly of interest is also the fact that vascularization of some particular tumors almost entirely rely on a single angiogenic factor, like bFGF in giant-cell tumors of the bone and angioblastomas. In addition, endogenous angiogenesis inhibitors like thrombospondins can also play an important role in the development of future inhibitors[118]. It has been shown that most angiogenesis inhibitors reach their maximal effectiveness when a constant product concentration is kept in the circulation[119].

4.5.4 Resistance to anti-angiogenic therapy

Although angiogenesis inhibitors have been shown to confer many survival benefits to patients bearing aggressive tumors, these compounds failed to produce a long-term clinical response. Anti-angiogenic treatment unfortunately results in only transitory improvements and after a while the tumors will inevitably start to grow again.

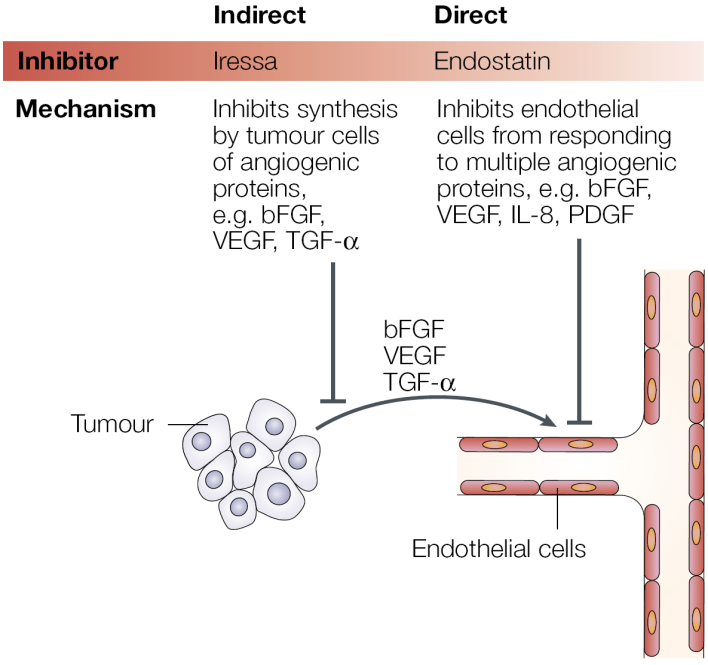


Figure 4. Mechanism of action of angiogenesis inhibitors[105]

4.5.4.1 Evasion of antiangiogenic therapy

One of the suggested mechanisms to explain the developing resistance to anti-angiogenic compounds is the concept of indirect evasive resistance, which is based on at least four different adaptations of molecular signaling pathways.

Activation of alternative pro-angiogenic pathways

In a mouse model of pancreatic neuroendocrine cancer, treatment with a monoclonal antibody targeting vascular endothelial growth factor receptor (VEGFR) caused an initial reduction in tumor vascularity followed by tumor regrowth. Higher levels of alternative pro-angiogenic factors like FGF 1 and 2, ephrin (EFN) A1 and A2 as well as angiopoietin 1 (ANGPT1), were found in relapsing tumors, suggesting that angiogenic factors other than VEGF were involved in restoring vascularization[120]. A similar mechanism of FGF induced revascularization, in particular FGF2, was also observed in a clinical study involving glioblastoma patients treated with the VEGFR inhibitor cediranib (Recentin, Astra Zeneca)[121]. These examples show how alternative angiogenic factors can take over once VEGF has been successfully inactivated.

Recruitment of vascular progenitor cells from the bone marrow

Tumor hypoxia can also trigger the recruitment of various bone-marrow derived cells (BMDCs) that have the power to initiate revascularization. Pro-angiogenic BMDCs comprise vascular progenitors, like endothelial cells and pericytes, as well as vascular modulators, like tumor associated macrophages[122] and immature monocytic cells. Vascular modulators exert their effects by releasing proteases, growth factors and cytokines[123, 124]. Extensive hypoxia and consequent up-regulation of HIF-1α were shown to promote angiogenesis due to recruitment of bone marrow derived $CD45^+$ myeloid cells in a model of glioblastoma multiforme (GBM). These effect was not seen in GBM lacking HIF-1α[124, 125]. Additional evidence supporting a role for hypoxia in BMDCs recruitment came from a study demonstrating that vascular disruption triggers accumulation of endothelial cells at the tumor margins[126].

Tumor vessel protection through increased pericyte coverage

Pericytes are also an important element of the tumor vasculature. It is known that tumor vessels lacking tight pericyte coverage are more vulnerable to anti-VEGF

therapy[92, 127]. Following impaired vascularization due to anti-angiogenic therapy some tumors rely on pericytes to protect and keep alive a core of functional blood vessels. Vessels, which survived inhibition of VEGF signaling, exhibited a different morphology being tightly covered with pericytes[127-132]. Pericytes are thought to protect the vessels by mediating endothelial cell quiescence and rendering them less responsive to therapy. Based on these data, combined targeting of pericytes and endothelial cells via inhibition of VEGF and platelet-derived growth factor (PDGF) respectively, was shown to improve efficacy of anti-angiogenic treatment in several mouse models and is currently tested in clinical trials[127, 133, 134]. Although the idea to dissociate pericytes from blood vessels to render them more susceptible to anti-VEGF therapy seemed to be a promising option, several studies showed that loss of pericyte coverage causes disruption of vascular integrity facilitating the transit of tumor cells into the circulatory system. As an example, increased metastasis as a result of pericyte targeting was observed in a murine model of pancreatic neuroendocrine tumor[135].

Increased invasion without angiogenesis

Another response to angiogenesis inhibition is an increased invasiveness of cancer cells. In a mouse model of GBM in which angiogenesis was inhibited either with VEGF inhibitor or genetically blocked by deletion of VEGF, HIF-1α or matrix metalloproteinase 9 (MMP9), tumors were shown to be more invasive[124, 136, 137]. These observations were recently confirmed in clinical studies involving GBM patients, who developed tumor recurrence following anti-VEGF therapy[138, 139]. This phenotype might be explained via activation of a pre-existing invasion program or via a switch to invasive growth due to nutrient deprivation.

4.5.4.2 Indifference to anti-angiogenic therapy

Resistance to anti-angiogenic therapy could also imply an intrinsic, already existing non-responsiveness of tumors. This was observed in a minority of patients, who did not respond at all to vascular targeting and whose tumors were continually growing during therapy[121].

Pre-existing multiplicity and redundancy

In contrast to early-stage breast cancers, late-stage breast tumors were found to express many angiogenic factors, including FGF2, different from VEGF. This expression pattern could explain, why an anti-angiogenic therapy targeting VEGF, would not be of benefit for late stage breast tumors, which switched already on a pre-existing alternative angiogenic program[140]. Nevertheless, anti-VEGF therapy was approved for the treatment of late-stage cancer diseases to achieve chemosensitization[129]. Vessel normalization and improved blood flow due to VEGF inhibition might in fact facilitate the diffusion of cytotoxic chemotherapeutics.

Pre-existing inflammatory mediated vascular protection

A recent study performed in mice showed that tumors with a large infiltration of inflammatory cells, secreting pro-angiogenic factors, are less responsive to anti-angiogenic therapies approaches compared to tumors having a less pronounced inflammatory cell infiltration. This effect was seen independently of a previous applied chemotherapy[141], suggesting a role for inflammatory cells and their secreted factors in vascular protection.

Characteristic hypovascularity

Some tumors like pancreatic ductal adenocarcinoma (PDAC) are characterized by a typical hypovascularization. Despite an avascular core, these tumors do not present necrosis suggesting an adaptation to the hypoxic microenvironment. This intrinsic tumor physiology might render PDAC indifferent to anti-angiogenic treatments. Clinical trials of anti-angiogenic therapy combined with chemotherapy seem to confirm the intrinsic resistance of hypovascular tumors to the treatment[142].

Invasion of normal vessels

A subset of tumors, namely grade II and III astrocytomas, fail to form lesions with a prominent vascularized core, where proliferation of endothelial cells takes place. Since these tumors do not develop the typical aberrant angiogenic vasculature typically targeted by anti-angiogenic compounds, they are suspected not to respond to classical anti-angiogenic therapy[143].

4.6 The role of HIF in vascularization and vascular remodelling

4.6.1 Angiogenesis and vascular remodelling

A role for HIF in angiogenesis is documented by several *in vivo* and *in vitro* studies. Although embryos of mice lacking HIF-1α expression normally start vasculogenesis, their initial vasculature was shown to degenerate leading to midgestation embryonic lethality, suggesting an impairment of angiogenesis or arteriogenesis[40, 144].

Expression of the most critical angiogenic factors including VEGF, stromal derived factor 1 (SDF1), ANGPT2, PlGF, PDGFB, and stem cell factor (SCF), was shown to be induced by hypoxia through upregulation of HIF-1 and to be cell-type specific[145-150]. While direct HIF-1 binding to promoter and activation of transcription has been shown for VEGF, SDF1 ANGPT2 and SCF genes[145-148], it is not yet clear if HIF-1 activates PlGF and PDGFB directly or indirectly. High induction of HIF-1 mRNA as well as HIF-1 protein levels followed by an increase in mRNA expression of several pro-angiogenic factors was observed in mouse ischemic limbs at day 3 after femoral artery ligation. These responses were impaired in HIF-1$\alpha^{+/-}$ animals[150].

Secreted angiogenic factors also play a role in the mobilization of pro-angiogenic cells from the bone marrow or from other tissues. This population of circulating angiogenic cells (CACs) includes endothelial progenitor cells (EPCs), hematopoietic stem-progenitor cells, mesenchymal stem cells (MSCs) as well as myeloid cells[151-155]. CACs are characterized by co-expression of specific receptors for angiogenic cytokines and progenitor cell markers. Because of the reduced expression of the angiogenic factors VEGF and SDF1 in ischemic tissue of HIF-1$\alpha^{+/-}$ mice, recruitment and mobilization of CACs was found to be impaired[150].

In contrast to the well studied stimuli resulting in angiogenesis, stimuli able to trigger arteriogenesis are less well understood. Although arteriogenesis occurs in non-hypoxic areas, it has been shown to occur concomitantly with angiogenesis after femoral arterial ligation[156-158]. In fact, proangiogenic myeloid cells need to pass through collateral remodelled blood vessels to reach the ischemic site. In addition, angiogenic factors like VEGF and PlGF were shown to be also arteriogenic[159, 160]. Vascular remodeling seems to be triggered by signals coming from ischemic tissues as well as from recruited bone marrow derived angiogenic cells (BMDAC). Current

knowledge suggests at least a partial overlap between signals triggering angiogenesis and signals inducing arteriogenesis, and there is increasing evidence for HIF-1 involvement in both processes[145, 147, 149, 150, 161, 162].

4.6.2 Effects of ageing and diabetes

Critical limb ischemia (CLI) is a disease characterized by decreased tissue perfusion that causes pain, ulceration and gangrene eventually resulting in limb amputation[163]. Similarly to CLI patients, impaired recovery of limb perfusion after experimental arterial ligation was also observed in older mice and in HIF-1$\alpha^{+/-}$ mice at all ages[150, 164]. Impaired wound healing is a consequence of diabetes mellitus in humans and was also observed in a diabetes type 2 mouse model[165]. Hypoxia-induced stabilization of HIF-1α in mouse fibroblasts is impaired after exposure to high glucose. Moreover, HIF-1α protein levels were found to be lower in diabetic wounds compared to non-diabetic ulcers. Lower levels of HIF-1α and proangiogenic HIF target genes were observed in excisional wounds of diabetes type 2 mice. Also amount and mobilization of CACs were reduced in diabetic mice[166-174].

4.6.3 Pathophysiological effects of HIF-1

High levels of HIF-1 were shown to be associated with highly vascularized and aggressive tumor phenotypes. Accordingly, loss of HIF-1 resulted in decreased tumor proliferation and angiogenesis[144]. Most cancer cells express high levels of VEGF and SDF1 due to HIF-1 upregulation as well as high mobilization and recruitment of CACs[124, 175-178].

In addition to their role in cancer, multiple proangiogenic HIF-1 target genes have been shown to be also implicated in retinal neovascularization, a pathological condition characterized by excessive blood vessel formation in the eye.

These results support a role for HIF-1 in diseases characterized by impaired vascularization.

4.7 PHDs and angiogenesis

In the last few years several studies addressed the question if prolyl-4-hydroxylases might be involved in angiogenesis by stabilizing HIF or even via HIF-independent pathways. First evidence was provided by the systemic application of prolyl-hydroxylase inhibitors, which were shown to promote angiogenesis through stabilization of HIF in endothelial and epithelial cells. The use of different PHD-inhibitors, namely L-mimosine (L-Mim), ethyl 3,4-dihydroxybenzoate (3,4-DHB), and 6-chlor-3-hydroxychinolin-2-carbonic acid-N-carboxymethylamid (S956711) was shown to induce HIF-α protein and HIF-target gene expression in human and rodent cells. Moreover, HIF-α induction in rat kidneys was observed after systemic administration of L-Mim or S956711[179]. The use of the selective HIF-PHD-inhibitor PHI-1 was shown to induce HIF-1α and HIF-2α in a cell culture model of bronchopulmonary dysplasia (BPD), a disease characterized by insufficient alveolar and vascular development. The treatment was shown to enhance VEGF secretion as well as glucose consumption and not to be toxic for human lung cells[180] . In a further study, the angiogenic phenotype of PHD1, PHD2 and PHD3 knock-out mice was evaluated. In Phd1 and Phd3 knock-out mice no visible angiogenic defects could be determined. In Phd2 knock-out mice, angiectasia, hyperactive angiogenesis and highly perfusable vessels were found. This angiogenic phenotype was independent of the local efficiency of PHD2 destruction in the single organs. Increased blood vessel density could be observed also in the brain, where the PHD2 knock-out was very inefficient compared to other organs like liver, heart, kidney and lung. These results suggest that PHD2 but not PHD1 or PHD3 is a major negative regulator of vascular growth in adult mice[81]. PHD2 silencing by RNA-interference (RNAi) was shown to be sufficient to increase HIF transcriptional activity in NIH3T3 cells[181]. Furthermore, *in vivo* angiogenesis could be observed in mice implanted with matrigel plugs containing NIH3T3 cells transfected with PHD2 short-interference (si)RNA constructs[181]. In a recent study, direct PHDs inhibition via short-hairpin (sh)RNA was shown to promote revascularization in a HIF-dependent manner in mice previously subjected to femoral artery ligation. These therapeutic revascularization was highest after PHD3 silencing, followed by PHD2 silencing, while lower proangiogenic activity was observed after PHD1 silencing[182]. Taken together, these data confirm a role for PHDs in modulating angiogenesis mainly by

stabilizing HIF expression and open new therapeutic possibilities for the treatment of ischemic diseases.

4.8 The role of HIFs in cancer biology

Solid human tumors usually present necrotic areas due to an insufficient blood supply. In addition to physical oxygen gradients within the tumor tissue, also temporal fluctuations in oxygen availability are common. Adaptation to the hypoxic tumor microenvironment promotes cancer progression, ultimately leading to death[183-185].

4.8.1 HIF-1α and HIF-2α levels in human cancers

In the majority of solid human tumors, immunohistochemical studies have shown increased levels of both HIF-1α and HIF-2α in the cancerous tissue compared to the adjacent normal tissue[186, 187]. Different factors including intratumoral hypoxia, presence of ROS and activation of the mitogen-activated protein (MAP) kinase pathways are responsible for the increased HIF-1 levels. In addition, HIF-1α and HIF-2α can be increased following genetic and epigenetic changes resulting in loss of function (LOF) of tumor suppressor genes or gain of function (GOF) of oncogenes. In cancer pathogenesis, cells with increased HIF activity seem to have a selective advantage[188-191].

4.8.2 Impact of HIF-1α and HIF-2α levels on cancer progression and mortality

A large amount of data support an association between high levels of HIF-1α or HIF-2α protein levels and increased patient mortality in many cancers. This relation is particularly well studied in breast cancer. A study involving 745 patients with invasive breast cancer showed that high HIF-1α levels correlated with poor overall survival as well as higher risk of metastasis formation[192]. A second study with 377

premenopausal breast cancer patients showed an impaired recurrence-free survival in lymph-node positive patients with high levels of HIF-1α [193]. A third study evaluated 132 patients with invasive breast carcinoma and again a positive significant correlation between HIF-1α and poor prognosis was found. In addition, also expression of the HIF- target gene carbonic anhydrase IX (CAIX) correlated in the same way, although being a weaker prognostic factor[194]. Similar conclusions were also drawn by Vleugel *et al*., who observed that perinecrotic HIF-1α overexpression accompanied by a strong induction of hypoxia-responsive genes like CAIX and glucose transporter 1 (GLUT1) associated with poor prognosis[195]. In a further study involving 171 breast cancer patients, increased HIF-1α correlated with shorter disease-free survival as well as overall survival[196]. Moreover, studies distinguishing between lymph-node positive or lymph node negative breast cancers, showed that HIF-1α is prognostic for an unfavourable outcome in both cases[197, 198]. Beside breast cancer, HIF-1α was also found to positively correlate with an aggressive colorectal carcinoma subtype, where its expression associated with increased metallothionein[199]. Interestingly, in a study involving 133 colorectal adenocarcinoma patients no correlation between clinical response and hypoxia markers, namely HIF-1α, HIF-2α, GLUT1 and CAIX expressed in the tumor cells was found. Conversely, a correlation between HIF-2α, GLUT1 and CAIX expressed in the tumor-associated stroma was observed. Furthermore, stromal HIF-2α and CAIX were both significantly associated with a reduced overall survival[200]. In 75 patients suffering from advanced head and neck squamous cell carcinoma (HNSCC) and treated with carboplatin chemoradiotherapy both HIF-1α and HIF-2α expression were found to correlate with poor local relapse-free survival and poor overall survival[201]. In non-small cell lung cancer (NSCLC) HIF-2α was shown to have a negative impact on overall survival while HIF-1α only marginally correlated with poor outcome. Both isoforms were shown to associate with increased expression of various angiogenic factors[202]. HIF-2α association with poor prognosis was also observed in malignant melanomas, where a high VEGF triggered angiogenic activity correlated with a limited inflammatory cell response[203]. Study of 58 PDAC patients showed significant correlation between HIF-1α and its target genes GLUT1 and VEGF with advanced tumor stage, lymph node metastasis and decreased overall survival[204]. In a recent study involving 90 patients suffering from rectal adenocarcinoma, HIF-1α but not

HIF-2α was found to correlate with a poor outcome[205]. A positive significant correlation between HIF-1α and VEGF levels with tumor grade, proliferation potential and vascular area was described in astrocytomas[206]. Taken together these studies suggest that HIF-α isoforms promote cancer progression and correlate with poor prognosis. However this conclusion is not absolute, as loss of HIF-2α was also shown to correlate with advanced tumor stage in immunohistochemical analysis of colon cancer biopsies[207]. Moreover, renal cell carcinoma xenografts suggest that HIF-1α GOF decreases xenograft growth[208].

4.9 PHDs and cancer

The role of prolyl-4-hydroxylases in cancer is still not well understood. Recent studies classified them as tumor suppressor genes, but their expression levels as well as their prognostic relevance appears to be highly heterogeneous. Although correlation studies with all three isoforms of PHD can be found in the literature, most of the efforts were done in unraveling the role of PHD2 in carcinogenesis. PHD3 was proposed as a tumor suppressor gene in colorectal carcinoma, where its decreased expression was found to correlate with higher tumor grade and metastasis. PHD3 seems to exert its tumor suppressor activity by inhibition of Iκκbeta phosphorylation and activation of NF-κB. Interestingly, this PHD3 effect was found to be independent of its hydroxylase function[209]. On the other hand, high nuclear expression of PHD1 and PHD3 was shown to associate with poorer survival in pancreatic endocrine tumors. In the same study, increased PHD2 expression was found to correlate with higher aggressiveness, higher rate of recurrence and death. In addition, cytoplasmic expression of FIH was found to be increased in pancreatic endocrine tumors[210]. Also in HNSCC increased expression levels and nuclear translocation of PHD2 were shown to be associated with less differentiated, strongly proliferating and therefore highly aggressive tumors. Moreover, in some HNSCC tumors high PHD2 expression was not sufficient to downregulate HIF. In line with these findings, low nuclear PHD2 expression in HNSCC was found to correlate with a positive outcome following radiation therapy[211, 212]. A complex model to correlate PHD2 levels with tumorigenesis was proposed by Lee *et al*. Their studies showed that in non-tumorigenic fibroblasts, a small decrease in PHD2 levels, obtained via RNAi, led to

malignant transformation, whereas severe downregulation of the enzyme did not. In line with these results, overexpression of PHD2 in malignant fibroblasts was found to inhibit their tumorigenic potential. From these data a biphasic model for PHD2 tumor forming potential was proposed[213]. Loss of PHD2 was found to increase tumorigenesis also in a colon cancer model. Tumors derived from PHD2 downregulated HCT116 cells were found to have a higher vessel density and to recruit more BMDCs. This PHD2 mediated angiogenic phenotype was found to be independent of HIF activity and PHD2 hydroxylation function. PHD2 downregulation was found to lead to angiogenin and interleukin-8 upregulation and this effect was shown to be NF-κB dependent[214]. In addition to studies investigating the role of PHD2 in tumor cells, recently also the effects of PHD2 levels in the surrounding environment particularly in endothelial cells, were considered. Analysis of the vasculature of tumors implanted in mice carrying a heterozygous PHD2 knock-out showed that in reduced PHD2 expression, vascular perfusion is improved due to vessel normalization and maturation. Interestingly, this normalization effect resulted in an inhibition of tumor invasion and metastasis[215].

5 Original publication

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ORIGINAL ARTICLE

Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis

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Hypoxia-elicited adaptations of tumor cells are essential for tumor growth and cancer progression. Although ample evidence exists for a positive correlation between hypoxia-inducible factors (HIFs) and tumor formation, metastasis and bad prognosis, the function of the HIF-α protein stability regulating prolyl-4-hydroxylase domain enzyme PHD2 in carcinogenesis is less well understood. In this study, we demonstrate that downregulation of PHD2 leads to increased tumor growth in a hormone-dependent mammary carcinoma mouse model. Tissue microarray analysis of PHD2 protein expression in 281 clinical samples of human breast cancer showed significantly shorter survival times of patients with low-level PHD2 tumors over a period of 10 years. An angiogenesis-related antibody array identified, amongst others, amphiregulin to be increased in the absence of PHD2 and normalized after PHD2 reconstitution. Cultivation of endothelial cells in conditioned media derived from PHD2-downregulated cells resulted in enhanced tube formation that was blocked by the addition of neutralizing anti-amphiregulin antibodies. Functionally, amphiregulin was regulated on the transcriptional level specifically by HIF-2 but not HIF-1. Our data suggest that PHD2/HIF-2/amphiregulin signaling has a critical role in the regulation of breast tumor progression and propose PHD2 as a potential tumor suppressor in breast cancer.
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Keywords: amphiregulin; hypoxia-inducible factors; mammary carcinoma; prolyl-4-hydroxylases; tumor vascularization

Introduction

Hypoxia is a characteristic feature of most solid tumors, contributes to the malignant phenotype and is associated with resistance to therapies and poor prognosis (Brown and Wilson, 2004; Pouysségur *et al*., 2006). Adaptive cellular responses to hypoxia are mainly mediated by heterodimeric hypoxia-inducible transcription factors (HIFs) and many HIF target genes are involved in cancer progression (Wenger, 2002; Semenza, 2003; Wenger *et al*., 2005). On the molecular level, HIF-α protein stability and transactivation activity are regulated by oxygen-dependent hydroxylation of specific prolyl and asparaginyl residues. The HIF prolyl-4-hydroxylase domain proteins, PHD1, 2 and 3 are responsible for normoxic HIF-α proteolysis. Prolyl-4-hydroxylation is necessary for the interaction with the von Hippel–Lindau tumor suppressor protein (pVHL) that mediates HIF-α degradation (Maxwell *et al*., 1999; Bruick and McKnight, 2001; Epstein *et al*., 2001; Jaakkola *et al*., 2001; Ivan *et al*., 2002).

Although HIF-α overexpression is found in most human cancers (Zhong *et al*., 1999) and correlates with poor prognosis and resistance to treatment (Aebersold *et al*., 2001), the role of PHDs in tumor formation remains incompletely understood. Tumor suppressor functions have been proposed for PHD1 as well as PHD3, whereas both tumor promoting as well as suppressing effects have been reported for PHD2. Ectopic expression of PHD1 in colon carcinoma cells inhibited tumor growth and correlated with increased necrosis and decreased microvessel density in a mouse xenograft model (Erez *et al*., 2003). PHD3 was found to be downregulated in colorectal cancer cells and correlated with higher tumor grade and metastasis (Xue *et al*., 2010) and loss of PHD3 was associated with the development of pheochromocytomas by acting downstream of c-Jun-mediated apoptosis (Lee *et al*., 2005). PHD2 has been reported to induce senescence in endometrial cancer cells by regulating HIF-mediated signal transduction pathways (Kato *et al*., 2006). On the other hand, increased expression levels of PHD2 have been shown to be associated with an aggressive phenotype in head and neck squamous cell carcinoma (Jokilehto *et al*., 2006). Overexpression of all three PHDs and the asparaginyl hydroxylase factor inhibiting HIF correlated with tumor aggressiveness and higher rate of recurrence in pancreatic endocrine tumors (Couvelard *et al*., 2008). In addition, a biphasic model for the relationship between PHD2 and tumor-forming potential has been suggested: small decreases of PHD2 led to malignant transformation of

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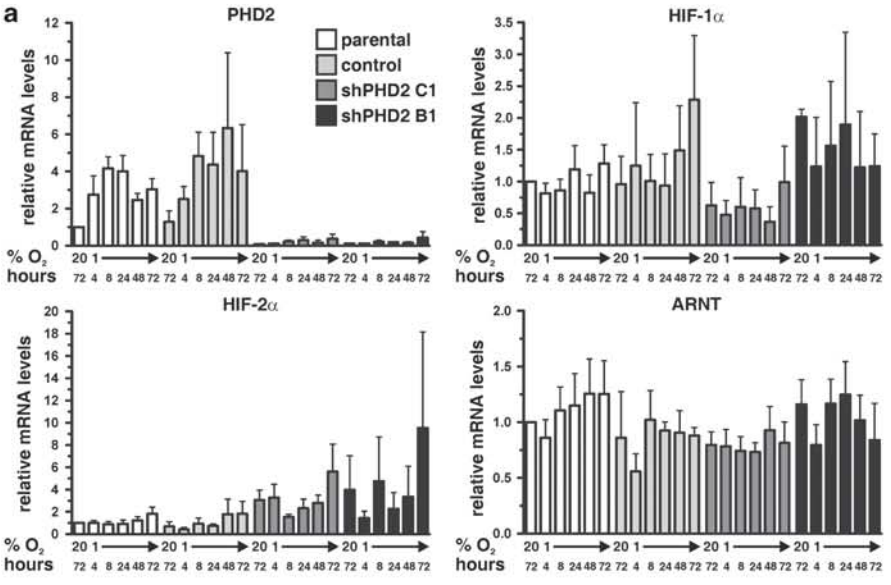
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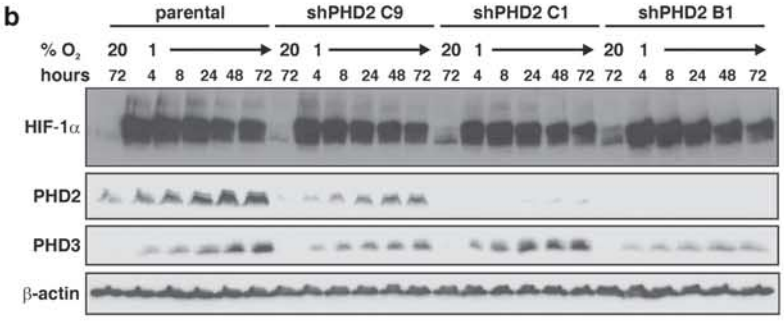
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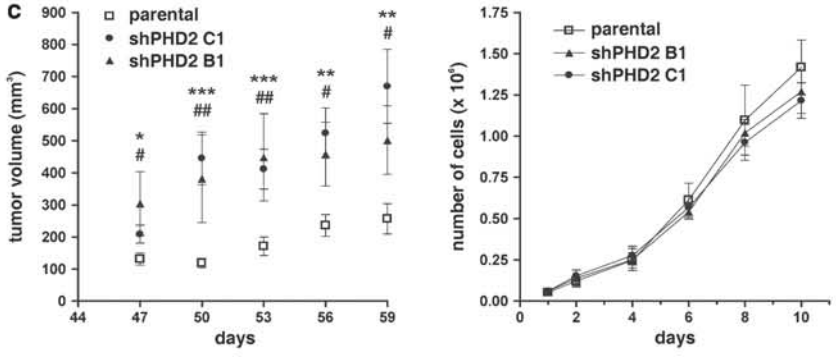
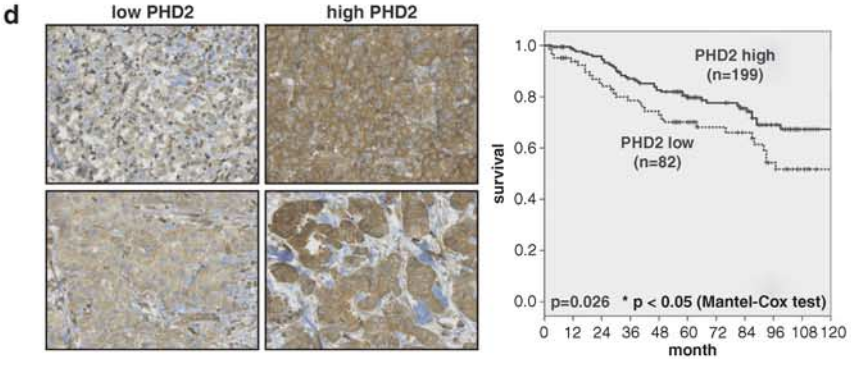

c
parental
shPHD2 C1
shPHD2 B1
tumor volume (mm3)
days
number of cells (x 10^4)
parental
shPHD2 B1
shPHD2 C1
days


d
low PHD2
high PHD2
PHD2 high
(n=199)
PHD2 low
(n=82)
survival
p=0.026 * p < 0.05 (Mantel-Cox test)
month

non-tumorigenic fibroblasts, whereas strongly decreased PHD2 levels did not (Lee *et al*., 2008). In summary, the function of PHDs in cancer remains incompletely understood. Current data suggest that they can be functionally inactivated, genetically mutated and epigenetically downregulated in cancer and additional tissue-specific factors might impinge on PHD regulation.

In this study, we have studied the function of PHD2 in a mammary carcinoma model and demonstrate that PHD2 downregulation promotes tumor growth. In clinical samples, low-PHD2 protein expression levels correlated significantly with shorter survival times of breast cancer patients. We confirmed PHD2-dependent vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) modulation and identified amphiregulin to be induced by loss of PHD2 and normalized by PHD2 reconstitution, suggesting that amphiregulin contributes to breast cancer progression.

Results

Increased tumor progression by PHD2 downregulation

To investigate the role of PHD2 in tumor development, stable PHD2 knockdown clones and non-targeting control clones were generated in human breast carcinoma MCF-7 cells by short hairpin RNA (shRNA) interference. After selection, stable shPHD2 clones C1 and B1 were further analyzed by reverse transcriptase quantitative PCR (RT–qPCR). Both clones showed efficient PHD2 mRNA downregulation under normoxic as well as prolonged hypoxic conditions (Figure 1a), whereas PHD1 and PHD3 mRNA levels were not significantly altered (data not shown). HIF-1α, HIF-2α and ARNT mRNA levels slightly varied under the conditions analyzed, but were not significantly affected by PHD2 downregulation (Figure 1a). Parental MCF-7 and control shRNA-transfected cells differed neither on the PHD2 mRNA (Figure 1a) or protein (Supplementary Figure S1) level, nor in proliferation or morphology (data not shown). Thus, both cell lines were subsequently used as controls. Immunoblot analysis confirmed efficient PHD2 knockdown in C1 and B1 clones also under prolonged hypoxic, that is, PHD2 inducing conditions (Figure 1b). Stabilization of normoxic HIF-1α and increased hypoxic accumulation of HIF-2α protein was observed in PHD2-downregulated MCF-7 cells (Figures 1b and 2b, respectively). In clone C9, the less efficient downregulation of PHD2 correlated with reduced normoxic HIF-1α stabilization when compared with the clones C1 and B1 (Figure 1b). Normoxic HIFα protein accumulation in shPHD2 cells is less pronounced than in hypoxic control cells, indicating that additional factors might contribute to the normoxic regulation of HIF-α subunits in MCF-7 cells.

To analyze the function of PHD2 in tumor formation *in vivo*, athymic nude mice were subcutaneously injected with parental and PHD2 knockdown MCF-7 cells. Because MCF-7 proliferation is estrogen dependent (Supplementary Figure S2), estrogen-releasing pellets were subcutaneously implanted into mice before the injection of MCF-7 cells. Tumor growth of PHD2-downregulated MCF-7 cells *in vivo* was significantly increased when compared with control cells (Figure 1c, left panel). Importantly, differences in tumor growth were independent of unequal intrinsic proliferation potentials, as parental and shPHD2 MCF-7 cells showed comparable growth rates and viability *in vitro* (Figure 1c, right panel). Tumor growth was analyzed over a time period of 60 days. Thereafter, sustained *in vivo* PHD2 suppression was confirmed on the mRNA as well as on the protein levels (Supplementary Figure S3). Recently, it has been proposed that heterozygous endothelial Phd2 deficiency stabilizes tumor vasculature and inhibits metastasis (Mazzone *et al*., 2009). Although tumors in our model were wild type for Phd2 in host-derived endothelial cells, we did not observe metastasis in mice subcutaneously injected with shPHD2 cells. Because MCF-7 are poorly invasive breast cancer cells, we injected these cells also intravenously but still detected no incidence of metastasis, even by RT–qPCR analysis of lung samples using human PHD2-specific primers (data not shown).

Analysis of PHD2 protein expression using a tissue micro array (TMA) of 281 human breast cancer patient samples showed predominantly a diffuse and fairly homogenous cytoplasmic immunoreactivity in invasive breast cancer cells and no membraneous staining. Low or very high PHD2 levels were observed in 82 and 199 cases, respectively (Figure 1d, left panel). Apart from a significant association of low PHD2 levels with a postmenopausal status, no other associations with clinicopathological parameters were found (Supplementary Table 1). Univariate survival analysis by the Kaplan–Meier method showed significantly shorter survival times of patients with tumors having low PHD2 levels (10 years survival rates: 52 vs 67%, $P=0.026$; Figure 1d, right panel). In a probatory multivariate Cox analysis (including tumor grades pT, pN, G) of estrogen receptor and PHD2, PHD2 failed to demonstrate independent prognostic significance (data not shown). To confirm the specificity of the anti-PHD2 antibody, parental MCF-7 and shPHD2 C1 cells were pelleted and embedded in paraffin for immunohistochemical detection of PHD2 protein expression using the same conditions as for the TMA experiments (Supplementary Figure S4).

Figure 1 PHD2 downregulation promotes tumor progression. (**a**) Transcript levels of PHD2, HIF-1α, HIF-2α and ARNT were analyzed in parental, control shRNA and shPHD2 MCF-7 cells by RT–qPCR. Cells were exposed to hypoxia (1% O_2) for up to 72 h. (**b**) Protein levels of HIF-1α, PHD2 and PHD3 were determined by immunoblotting. (**c**) Parental and shPHD2 MCF-7 cells were subcutaneously injected into nude mice and tumor growth measured over 60 days starting from a minimal tumor diameter of 5 mm (left panel). *In vitro* cell proliferation of parental and shPHD2 MCF-7 cells was assessed by counting cells over 10 days (right panel). (**d**) Examples from mammary carcinoma TMA immunostaining for PHD2 (left panel) and ten years survival analysis by Kaplan–Meier plotting (right panel). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, C1 vs parental; $^{\#}P<0.05$, $^{\#\#}P<0.01$, B1 vs parental.

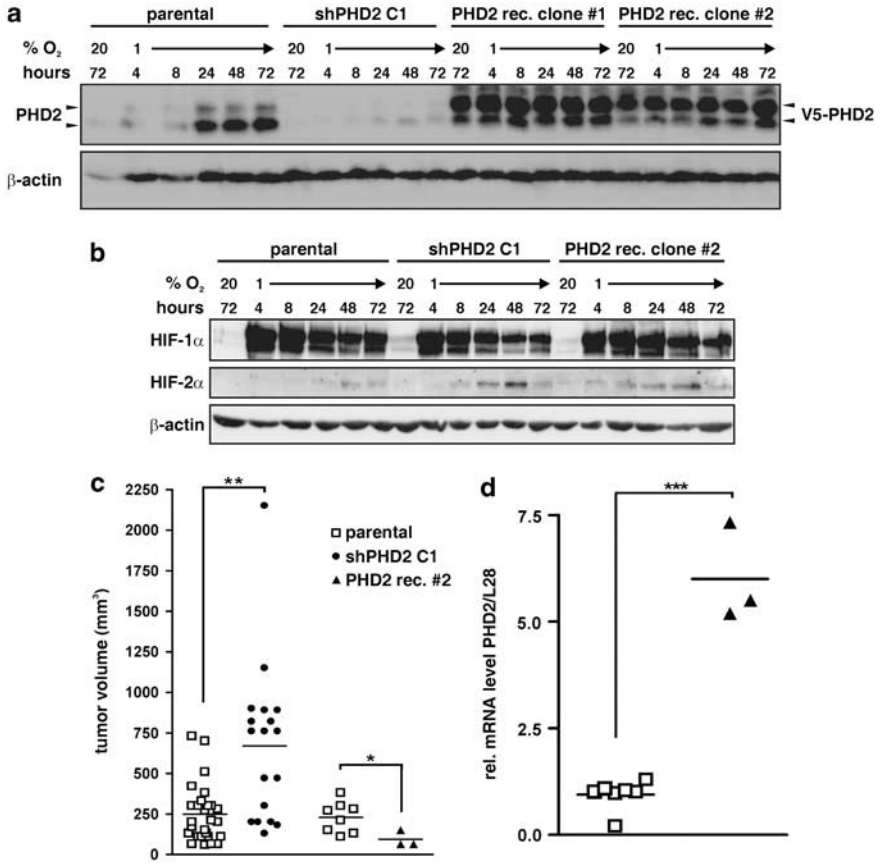


Figure 2 Tumor growth is repressed by PHD2 overexpression. (**a**) MCF-7 shPHD2 cells were transfected with a V5-tagged PHD2 expression vector and PHD2 protein levels determined by immunoblotting. (**b**) Protein levels of HIF-1α and HIF-2α were determined by immunoblotting. (**c**, **d**) Parental, shPHD2 C1 and PHD2-reconstituted MCF-7 cells (clone no. 2) were subcutaneously injected into nude mice and tumor volume (**c**) as well as PHD2 mRNA levels (**d**) determined at the end of the experiment after 60 days. Rec., reconstituted, Rel., relative. $*P<0.05$, $**P<0.01$, $***P<0.001$.

Overexpression of PHD2 in shPHD2 cells suppresses tumor growth

To exclude that shRNA off-target effects increased the tumor growth in shPHD2 MCF-7 clones, we reconstituted the expression of PHD2 in the stable shPHD2 clone C1. Immunoblot analysis showed constitutively elevated PHD2 protein levels under normoxic as well as hypoxic conditions in the selected reconstituted clones no. 1 and no. 2 (Figure 2a). PHD2 reconstitution also reduced normoxic HIF-1α protein levels and attenuated hypoxic HIF-2α accumulation (Figure 2b). Although PHD2 expression was higher in both reconstituted clones than in parental MCF-7 cells, no difference in *in vitro* proliferation rates and viability was observed (data not shown). Implantation of PHD2-reconstituted and parental MCF-7 cells into athymic nude mice resulted in tumors in 13.6 and 77.3% of all cases, respectively. In addition, the three successfully grown tumors of PHD2-reconstituted cells showed a significantly decreased volume compared with tumors derived from parental MCF-7 cells (Figure 2c). Subsequent tumor tissue analysis confirmed increased PHD2 expression in reconstituted compared with parental cells, suggesting that PHD2 overexpression leads to suppression of tumor growth (Figure 2d).

PHD2 suppression increases tumor angiogenesis and modulates secreted angiogenic factors

Tumor vascularization of experimental tumors derived from parental and shPHD2 MCF-7 cells was analyzed by PECAM-1 immunostaining. Knockdown of PHD2 resulted in a more elaborated and denser vascular network (Figure 3a). Quantitative analysis revealed a significant increase in vessel density, area and diameter in shPHD2 compared with parental cells (Figure 3b). Tumors derived from parental or shPHD2 MCF-7 cells showed no obvious morphological differences by hematoxylin/eosin staining (data not shown).

To investigate a possible regulation of secreted angiogenic factors by altered PHD2 levels, we incubated human angiogenesis arrays with conditioned media of parental, shPHD2 or reconstituted PHD2 MCF-7 cells. To eliminate potential clonal differences between maternal and shPHD2 cell lines, we also used pools of lentivirally transduced shPHD2 MCF-7 cells (Supplementary Figure S5). Compared with conditioned media of parental MCF-7 and reconstituted PHD2 cells, VEGF was increased in shPHD2 cells, suggesting activation of the HIF pathway (Supplementary Figure S6). In addition, IL-8 as well as amphiregulin was elevated in the absence of PHD2, whereas reduced in

cells overexpressing PHD2 when compared with parental MCF-7 cells. Recently, IL-8 has been shown to be induced by PHD2 downregulation in a HIF-independent but nuclear factor-κB (NF-κB)-dependent manner (Chan *et al*., 2009), confirming the relevance of our experimental approach. However, amphiregulin induction by PHD2 downregulation has not been described previously.

Other pro-angiogenic factors abundantly secreted by MCF-7 cells include insulin-like growth factor binding protein 2, matrix metallopeptidase 9 and tissue inhibitor of metalloproteinase 1. However, secretion of these factors was independent of PHD2 (Supplementary Figure S6).

PHD2 regulates amphiregulin-dependent tube formation in vitro

To elaborate the PHD2-dependent function of amphiregulin in more detail, we performed tube formation assays *in vitro*. Incubation of HUVEC cells with supernatant of parental MCF-7 cells resulted in moderate tube formation (Figure 3c). In contrast, after PHD2 downregulation tube formation was significantly increased as determined by counting branch points and estimating total tube network length. Conversely, tube formation was markedly decreased by shRNA-mediated downregulation of amphiregulin gene expression (Figure 3c), already following amphiregulin transcript level repression by only approximately 50% (Supplementary Figure S7). Importantly, stimulation of tube formation by supernatants of shPHD2 MCF-7 cells was significantly reduced by pre-incubation with a neutralizing anti-amphiregulin antibody (Figure 3d), suggesting that the PHD2-mediated increase in tube formation was at least partly mediated by amphiregulin secretion.

Amphiregulin transcription is regulated by PHD2

Analysis of amphiregulin mRNA levels in MCF-7 cells by RT–qPCR demonstrated increased amphiregulin transcript levels after PHD2 suppression and reduced amphiregulin mRNA levels after PHD2 overexpression (Figure 4a). Transfection of a firefly luciferase reporter gene driven by the amphiregulin gene (*AREG*) promoter (pAREG) into parental, shPHD2 or reconstituted MCF-7 cells resulted in relative luciferase activities recapitulating the regulation of the endogenous amphiregulin transcript levels in these cell lines (Figure 4b). In contrast to the known hypoxia-inducible gene carbonic anhydrase 9 (CA9), amphiregulin mRNA levels were not induced under hypoxic conditions (Figure 4c), indicating that amphiregulin regulation might be independent of the prolyl-4-hydroxylase enzymatic function of PHD2. Moreover, pharmacologic inhibition of PHD hydroxylase function using the pan-PHD inhibitor DMOG, did not induce amphiregulin expression, whereas CA9 and PHD2 were increased as expected (Supplementary Figure S8).

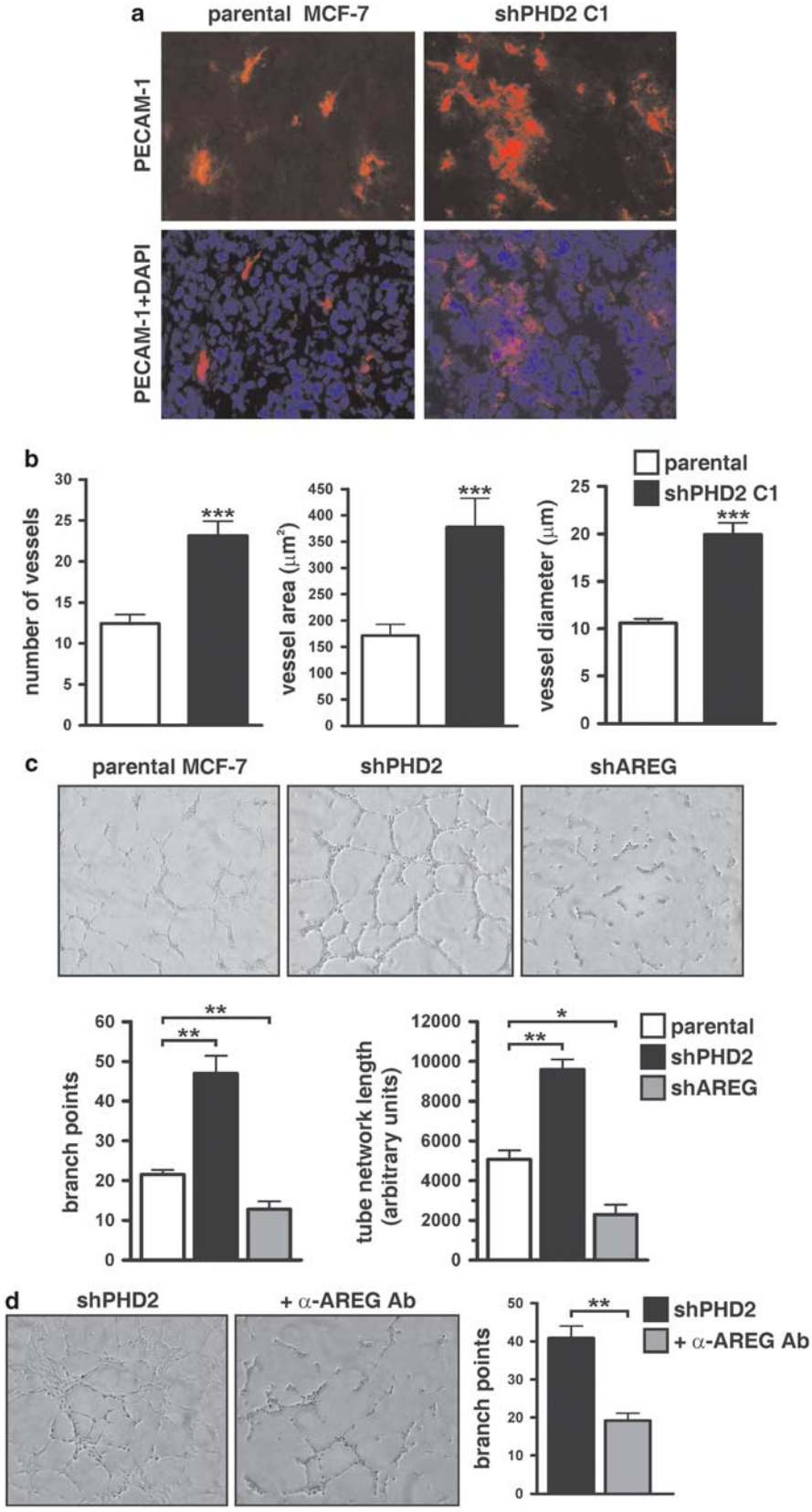
To analyze the function of PHD2 on amphiregulin transcription in more detail, we transfected shPHD2 MCF-7 cells with pAREG reporter plasmids (depicted in Figure 4d) together with active (PHD2) or inactive hydroxylase mutant PHD2. All pAREG reporter plasmids were similarly responsive to PHD2 coexpression, irrespective of the presence of the putative HIF-binding site and functional PHD2 hydroxylase activity (Figure 4e, left panel). As expected, the HIF-dependent reporter plasmid pH3SVL (Wanner *et al*., 2000) responded to wild type but not mutant PHD2. Hypoxic exposure only marginally induced pAREG reporter activity and the repressive effect of PHD2 coexpression was preserved at reduced oxygen concentration (Figure 4e, right panel). Note that pH3SVL was strongly induced by hypoxia and PHD2 reconstitution less efficiently reduced pH3SVL reporter activity under hypoxic than normoxic conditions. In conclusion, these data are all consistent with the notion that PHD2 represses amphiregulin transcription in a hydroxylase-independent manner.

Cyclic AMP response element (CRE)-binding protein-dependent regulation of amphiregulin gene expression has been reported in the intestinal epithelial cells and a functional CRE has been identified in the human amphiregulin promoter (Plowman *et al*., 1990; Lee *et al*., 1999; O'Reilly *et al*., 2006). To delineate the function of CRE-binding protein in PHD2-dependent pAREG regulation, we cotransfected shPHD2 MCF-7 cells with wild-type or mutant CRE pAREG reporter plasmids together with active or mutant PHD2. Although pAREG reporter activity was reduced on CRE site mutation, the wild-type and mutant PHD2 repressive effect were preserved (Figure 4f, left panel). The HIF-dependent reporter plasmid pH3SVL was again used as control (Figure 4f, right panel).

Whereas functional NF-κB-binding sites in the angiogenin as well as IL-8 promoters have been reported to be required for tumor necrosis factor-α-mediated induction of reporter gene expression (Chan *et al*., 2009), amphiregulin mRNA levels were neither induced by tumor necrosis factor-α treatment (Supplementary Figure S9) nor suppressed by small interfering RNA (siRNA)-mediated downregulation of the NF-κB subunit RelA (Supplementary Figure S10). These data suggest that the signaling pathway involved in the PHD2-dependent control of amphiregulin expression in mammary carcinoma cells is different from the signaling pathway reported for PHD2- and NF-κB-dependent regulation of angiogenin and IL-8 in colon carcinoma cells (Chan *et al*., 2009).

HIF-2α-specific regulation of amphiregulin gene expression

Downregulation of PHD2 in MCF-7 cells resulted in a slight normoxic accumulation of both HIF-1α and HIF-2α protein levels (Figure 5a). Even though amphiregulin transcriptional regulation was independent of PHD2 hydroxylase function, we hence investigated whether amphiregulin expression might additionally be regulated in a HIF-α isoform-specific manner. Therefore, stable shHIF-1α and shHIF-2α MCF-7 pool of


a
parental MCF-7
shPHD2 C1
PECAM-1
PECAM-1+DAPI
b
parental
shPHD2 C1
number of vessels
vessel area (μm²)
vessel diameter (μm)
***
c
parental MCF-7
shPHD2
shAREG
branch points
tube network length (arbitrary units)
parental
shPHD2
shAREG
**
*
d
shPHD2
+ α-AREG Ab
branch points
shPHD2
+ α-AREG Ab

clones were generated (Figure 5b, left panel) and analyzed for amphiregulin gene expression by RT–qPCR (Figure 5b, right panel). Compared with parental cells, transcript levels of amphiregulin were upregulated in shHIF-1α and decreased in shHIF-2α MCF-7 cells, indicating that HIF-2α is mainly responsible for the regulation of amphiregulin gene expression. As evidenced by immunoblot analysis (Figure 5b, left panel), increased amphiregulin mRNA levels in shHIF-1α cells might probably be due to higher HIF-2α levels in these cells. Conversely, overexpression of HIF-2α in parental MCF-7 cells increased amphiregulin promoter-dependent reporter gene activity, whereas exogenous expression of HIF-1α had no effect (Figure 5c, left panel).

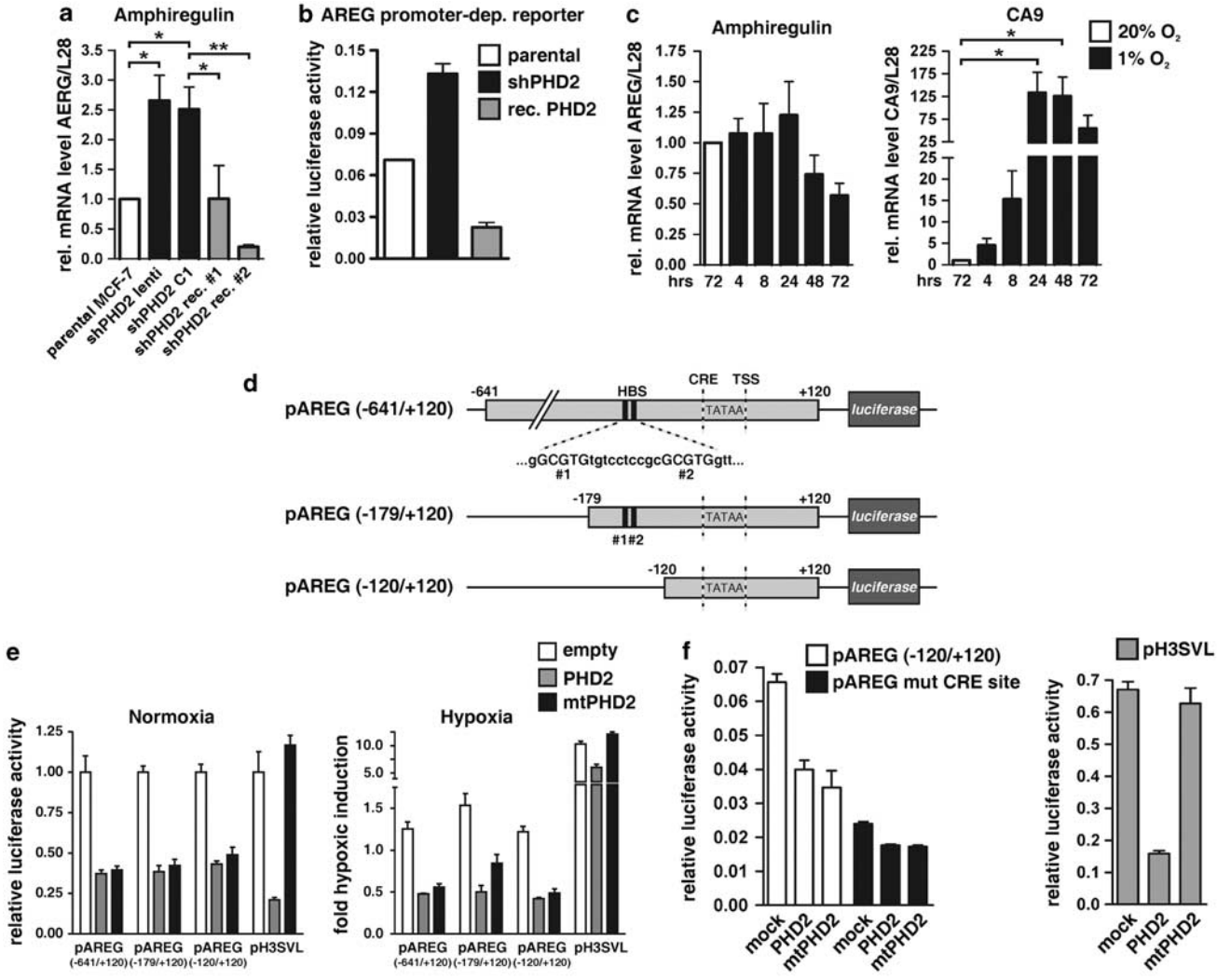


Figure 4 PHD2-dependent transcriptional regulation of amphiregulin. (**a**) Amphiregulin mRNA levels of parental, shPHD2 and PHD2-reconstituted MCF-7 cells were measured by RT–qPCR. (**b**) The amphiregulin promoter-driven firefly luciferase reporter gene construct pAREG(−640/+120) was transfected into parental, shPHD2 and PHD2-reconstituted MCF-7 cells and relative luciferase activities were quantified. (**c**) Amphiregulin and CA9 mRNA levels were measured by RT–qPCR in MCF-7 cells cultured under normoxic (20% O_2) or hypoxic (1% O_2) conditions for up to 72 h. (**d**) Schematic representation of the reporter gene constructs driven by the human *AREG* promoter. 5′-Regulatory elements are labeled relative to the transcriptional start site (TSS). Two putative consensus HIF binding sites (HBS) are indicated in capital letters. (**e**) A measure of 200 ng pAREG reporter constructs as indicated were cotransfected with either 800 ng wild-type PHD2 or a hydroxylase-deficient PHD2 mutant (mtPHD2), and relative luciferase activities were quantified. The HIF-dependent reporter plasmid pH3SVL served as control. (**f**) The indicated plasmids were cotransfected as mentioned above (**e**). dep., dependent; Rec., reconstituted, Rel., relative. $*P<0.05$, $**P<0.01$.

Figure 3 Tumor angiogenesis is enhanced by PHD2 suppression and subsequent amphiregulin upregulation. (**a**) PECAM-1 staining of cryosections derived from parental and shPHD2 MCF-7 tumors. (**b**) Quantification of vessel number, area and diameter. (**c**) HUVEC cells were resuspended in supernatants of parental, shPHD2 or shAREG MCF-7 cells and plated on matrigel-coated slides. Tube formation was quantified by estimating the number of branching points and overall length of the network. (**d**) HUVEC cells were resuspended in supernatants of shPHD2 cells containing a neutralizing antibody against amphiregulin and the branching points were counted. DAPI, 4′,6-diamidino-2-phenylindole, PECAM-1, platelet/endothelial cell adhesion molecule 1. $*P<0.05$, $**P<0.01$, $***P<0.001$.

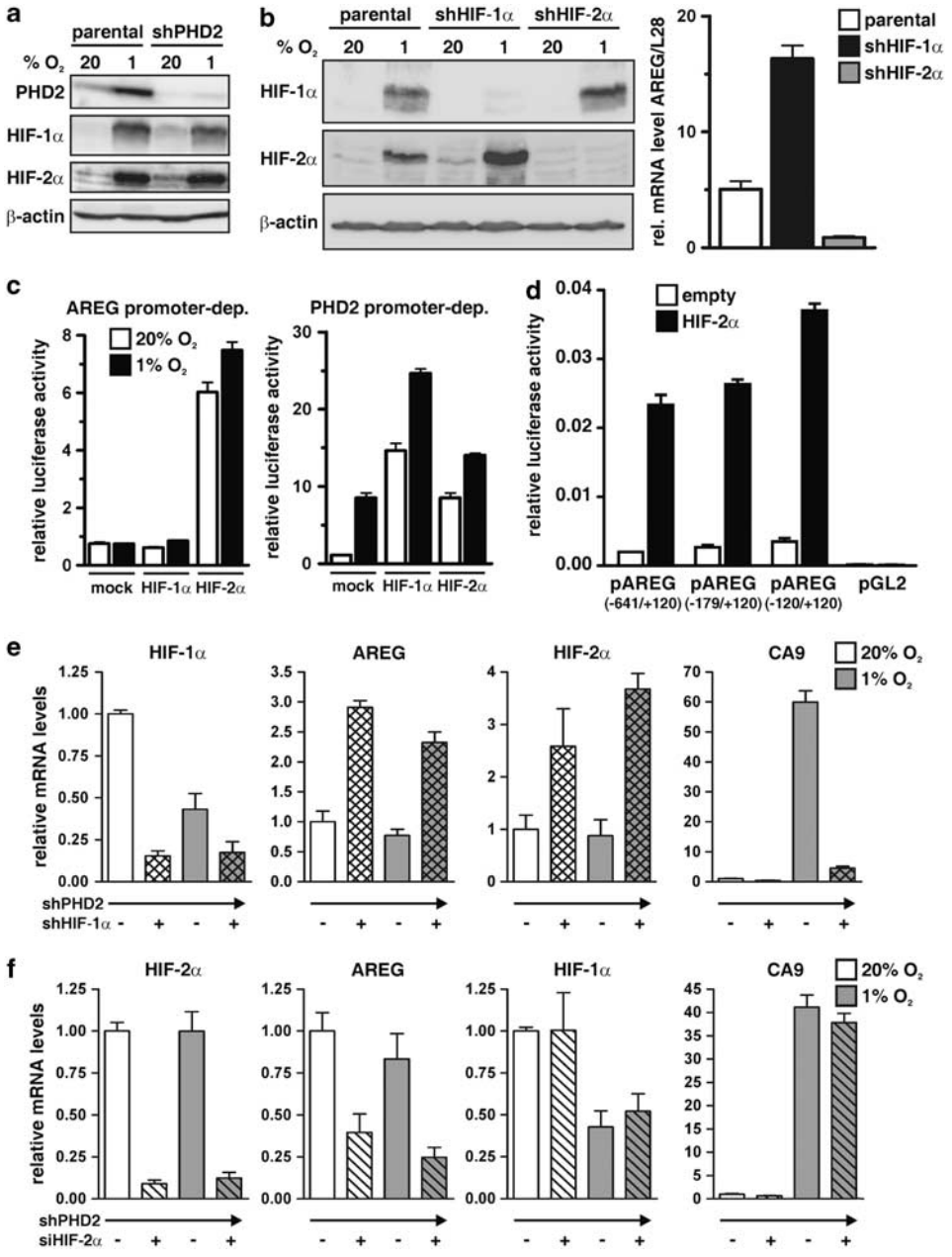


Figure 5 Amphiregulin transcription is regulated by HIF-2. (**a**) Protein levels of PHD2, HIF-1α and HIF-2α were determined by immunoblotting in parental and shPHD2 (clone C1) MCF-7 cells cultured under normoxic or hypoxic conditions. (**b**) Nuclear HIF-1α and HIF-2α protein levels were determined by immunoblotting (left panel) and amphiregulin mRNA levels were measured in parental, shHIF-1α and shHIF-2α MCF-7 cells (right panel). (**c**) MCF-7 parental cells were cotransfected with the amphiregulin promoter construct pAREG(−640/+120) together with either an empty, HIF-1α or HIF-2α expression vector. Cells were cultured under normoxic or hypoxic conditions for 24 h and relative luciferase activities quantified. A PHD2 promoter-driven reporter plasmid was used as a control. (**d**) MCF-7 shHIF-2α cells were cotransfected with 200 ng pAREG reporter construct as indicated together with either 800 ng HIF-2α or empty expression vector, and cultured under normoxic conditions. Promoterless pGL2basic served as negative control. Transcript levels of HIF-1α, HIF-2α, amphiregulin and CA9 were analyzed in shPHD2/shHIF-1α (**e**) and shPHD2/siHIF-2α (**f**) MCF-7 cells by RT–qPCR. dep., dependent; Rel., relative.

A PHD2 promoter-driven, that is, HIF-dependent, reporter plasmid was used to control for efficient HIF-1α and HIF-2α expression (Figure 5c, right panel).

To analyze the function of HIF-2 in amphiregulin promoter regulation, we used again the pAREG reporter constructs depicted in Figure 4d and cotransfected them into MCF-7 shHIF-2α cells together with either HIF-2α or an empty expression vector. All pAREG reporter plasmids were induced by exogenous HIF-2α expression, irrespective of the presence of the putative HIF-binding site (Figure 5d). The promoter-less reporter backbone pGL2 served as negative control.

Finally, either HIF-1α or HIF-2α was downregulated in shPHD2 MCF-7 cells and endogenous gene expression was analyzed by RT–qPCR. HIF-1α suppression upregulated amphiregulin mRNA levels (Figure 5e), probably indirectly because of elevated *HIF-2α* gene expression as previously observed on the protein level in stable HIF-1α knockdown cell pools (Figure 5b). In contrast, HIF-2α suppression downregulated amphiregulin mRNA levels and did not influence *HIF-1α* gene expression (Figure 5f). *CA9* served as positive control and seems to be a HIF-1-specific target gene. In conclusion, these data indicate that amphiregulin transcription is regulated by HIF-2 independent of the putative HIF-binding site in the promoter region.

Discussion

Although the role of HIFs in tumor formation and metastasis is well known, the function of regulators of HIF-α stability during neoplastic growth has come into focus only recently. We report in this study that PHD2 functions as a tumor suppressor in xenografted tumors derived from breast carcinoma MCF-7 cells and clinical data indicate that high PHD2 protein levels are a positive prognostic survival factor in human mammary carcinomas. Mechanistically, amphiregulin contributed to PHD2 regulated *in vitro* tube formation and amphiregulin transcription was increased specifically by HIF-2 in breast carcinoma MCF-7 cells.

During progression of our analysis, RNA interference-mediated downregulation of PHD2 has recently been used to further elucidate the function of PHD2 in tumor formation. Wu *et al*. (2008) observed elevated *in vivo* angiogenesis of tumors derived from PHD2-silenced NIH3T3 cells and this phenotype was attributed to HIF-mediated increased expression of the pro-angiogenic VEGF and fibroblast growth factor-2. Evidence for a tumor suppressive function of PHD2 has recently also been reported by Chan *et al*. (2009). PHD2 downregulation increased normoxic NF-κB activity, leading to a HIF-independent elevated secretion of the pro-angiogenic factors IL-8 and angiogenin in a colon carcinoma cell model. Immunohistochemical analysis of PHD2 protein levels in human breast cancer samples showed that 6 out of 11 patient samples had higher levels of PHD2 in normal tissue than in matched tumor samples (Chan *et al*., 2009). Our analysis of ten years survival rates from 281 clinical mammary carcinoma samples revealed significantly enhanced survival times of patients having tumors with high PHD2 levels, supporting the proposed tumor suppressing role of PHD2. Although high PHD2 transcript levels have been reported in estrogen receptor-negative and human epidermal growth factor receptor-2 (HER2)-negative breast carcinoma (Zhang *et al*., 2009), we found a significant association of low PHD2 levels with a postmenopausal state but not with tumor grades, estrogen receptor, progesteron receptor or HER2. Clinicopathological data from our cohort were collected before the broad introduction of aromatase inhibitors for postmenopausal women, but association of PHD2 with postmenopausal state might still be explained by estrogen receptor status alteration, because PHD2 correlated with fatty acid synthase, another marker of postmenopausal status. In tumor angiogenesis, PHD2 levels have to be considered not only in cancer cells but also in stromal cells. Indeed, endothelial PHD2 levels have been reported to dramatically affect tumor growth by influencing intratumoral vessel development (Mazzone *et al*., 2009). In summary, accumulating evidence suggests an important regulatory role of PHD2 in tumor angiogenesis.

Amphiregulin is a heparin-binding glycoprotein that has originally been isolated from conditioned media of human breast carcinoma MCF-7 cells and identified as epidermal growth factor receptor ligand (Shoyab *et al*., 1988). During development, amphiregulin is the most abundant epidermal growth factor-like growth factor in the pubertal mammary gland and has an important role in ductal morphogenesis (Luetteke *et al*., 1999). There is clear evidence for a role of amphiregulin in breast cancer initiation as well as progression (McBryan *et al*., 2008; Willmarth and Ethier, 2008). Amphiregulin is often overexpressed in mammary carcinoma (Qi *et al*., 1994) and generally higher in invasive breast carcinomas than in normal mammary epithelium (Panico *et al*., 1996).

Importantly, amphiregulin antisense expression in a transformed human breast epithelial cell line that has been selected for its increased tumorigenicity *in vivo*, reversed the malignant phenotype in nude mice and led to a significant reduction in tumor mass (Ma *et al*., 1999). Moreover, tumor vascularization was markedly reduced in tumors derived from amphiregulin antisense expressing cells, suggesting that amphiregulin modulates angiogenesis in these cells. Although it remains largely unknown how amphiregulin might mechanistically contribute to breast cancer pathogenesis in general, regarding angiogenesis it has been shown that amphiregulin antisense expression reduced the angiogenic factor TGFβ1 (Giusti *et al*., 2003) and upregulated the expression of IL-8 in human airway epithelial cells (Chokki *et al*., 2006). Our results showed that the *in vitro* tube formation potential of shPHD2 cells was significantly increased compared with parental MCF-7 cells and again reduced after addition of neutralizing anti-amphiregulin antibodies, although elevated levels of the potent angiogenic factors VEGF and IL-8 remained unchanged. This suggests that amphiregulin contributes substantially to pro-angiogenic modulation by MCF-7 breast cancer cells.

A few regulators of amphiregulin gene expression have been reported previously: EGF-related growth factors auto- and cross-induce each other (Barnard *et al*., 1994), and amphiregulin transcription is induced by estrogen, probably indirectly as no estrogen-response elements have been identified in the amphiregulin promoter region (Martinez-Lacaci *et al*., 1995). In fetal kidney differentiation, the zinc-finger transcription factor Wilms tumor suppressor (WT1) has been shown to regulate amphiregulin gene expression (Lee *et al*., 1999), but we did not find any WT1 expression in

MCF-7 cells (data not shown). Recently, BRCA1 has been described as transcriptional repressor of amphiregulin and loss of BRCA1 resulted in elevated amphiregulin protein levels (Lamber *et al*., 2010). The role of oxygen in amphiregulin regulation is incompletely understood. Whereas hyperoxia induced amphiregulin in a model of bronchopulmonary dysplasia (Wagenaar *et al*., 2004), hypoxia has been reported to upregulate amphiregulin gene expression in a CRE-binding protein-dependent manner in intestinal epithelial cells (O'Reilly *et al*., 2006).

Our results suggest that PHD2 regulates amphiregulin on the transcriptional level, directly or indirectly involving HIF-2 but not HIF-1 (a schematic overview is shown in Supplementary Figure S11). Intriguingly, although HIF-2 clearly increased endogenous amphiregulin mRNA levels and exogenous amphiregulin promoter activity, cultivation of MCF-7 cells under hypoxic conditions did not significantly regulate endogenous amphiregulin transcript levels or promoter activity. These data suggest that normoxic, basal HIF-2 levels are sufficient for amphiregulin gene expression which is not further induced by hypoxic stabilization of HIF-2α. Supporting this hypothesis, it has been shown that HIF-2 is slightly more abundant in normoxic and mildly hypoxic cells than HIF-1 (Wiesener *et al*., 1998) and a recent report demonstrated that the HIF-2-specific target gene, *CITED2,* was maximally expressed under normoxic conditions (Franovic *et al*., 2009). Furthermore, it has been shown that the vascular endothelial–cadherin gene is not regulated by hypoxic conditions, but the vascular endothelial–cadherin promoter is nevertheless activated by HIF-2 together with the Ets-1 transcription factor (Le Bras *et al*., 2007).

Several groups reported on the role of PHDs in regulating *in vivo* angiogenesis. Conditional knockout of PHD2, but not PHD1 or PHD3, resulted in increased secretion of VEGF and enhanced angiogenesis (Takeda *et al*., 2007) and application of pan-PHD inhibitors in rats led to HIF-α stabilization and stimulated angiogenesis (Warnecke *et al*., 2003). As PHD inhibitors have been developed to treat ischemic diseases, concerns might arise about adverse effects of these inhibitors in tumor progression. Our study demonstrates that PHD2 has a critical role in tumor progression, suggesting that a combinatory treatment regimen of PHD inhibitors together with antiangiogenic compounds or, if available, anti-HIF-α isoform-specific inhibitors, might be required to avoid the induction of potentially tumor promoting signaling pathways.

Materials and methods

Plasmids
Cloning of PHD2 expression vectors and other plasmids was carried out using Gateway technology (Invitrogen, Basel, Switzerland) as described previously (Barth *et al*., 2007).

Cell culture and transfections
Human MCF-7 breast carcinoma cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Sigma, Buchs, Switzerland) as described previously (Camenisch *et al*., 1999). HUVEC cells were kindly provided by TF Lüscher (Zürich, Switzerland) and cultured in complete endothelial basal medium-2 containing 2% fetal calf serum (Lonza, Basel, Switzerland). For long-term hypoxia, cells were grown in a gas-controlled glove box (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK). Transfections were performed using polyethylenimine (Polysciences, Warrington, PA, USA) as described previously (Stiehl *et al*., 2006).

Immunoblotting
Immunoblot analyses were performed as previously described (Balamurugan *et al*., 2009). Antibodies used were mouse monoclonal antibody (mAb) anti-HIF-1α (Transduction Laboratories, BD Biosciences, Allschwil, Switzerland), anti-HIF-2α (Novus Biologicals, LuBioScience, Lucerne, Switzerland), mAb anti-V5 (Invitrogen), mAb anti-β-actin (Sigma), rabbit polyclonal anti-PHD2 antibody (Novus), mAb anti-PHD3 was kindly provided by PJ Ratcliffe (Oxford, UK).

Reporter gene assays
Construction of the HIF-dependent firefly luciferase reporter gene construct pH3SVL was described previously (Wanner *et al*., 2000). The amphiregulin promoter plasmid pAREG (−641/+120) was kindly provided by SB Lee (Bethesda, USA) (Lee *et al*., 1999). Truncations of this plasmid have been constructed by PCR and recombinant cloning techniques. As sequencing revealed point mutations in the parental plasmid, the (−120/+120) region was *de novo* cloned from MCF-7 genomic DNA. The consensus CRE site (5′-TGACGTC-3′) in pAREG(−120/+120), located 65 nucleotides upstream of the transcriptional start site, was mutated to (5′-TGAaaTC-3′) by site-directed mutagenesis. The PHD2 luciferase reporter plasmid pP2P(−603/+3)wt is a truncated version of the previously published human PHD2 promoter (Metzen *et al*., 2005). Cells were cotransfected with 200 ng reporter plasmid and 10 ng pRLSV40 Renilla luciferase reporter vector (Promega, Dübendorf, Switzerland) and luciferase reporter gene activity was determined using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega).

mRNA quantification
Total cellular RNA was extracted as described previously (Barth *et al*., 2007). First-strand complementary DNA synthesis was performed with 1–5 μg total RNA using reverse transcriptase and mRNA levels were measured by real-time quantitativePCR using a SybrGreen qPCR reagent kit (Sigma) in combination with the MX3000P light cycler (Stratagene, Amsterdam, The Netherlands). To verify RNA integrity and equal input levels, ribosomal protein L28 mRNA was determined, and the data were expressed as ratios relative to L28 levels.

Mice xenografts
Estrogen-releasing pellets (0.72 mg 17β-estradiol 60-days release, Innovative Research of America, Sarasota, FL, USA) were subcutaneously implanted into the neck of NMRI female nude mice (Harlan Europe, The Netherlands) 5 days before subcutaneous injection of $1–2 \times 10^7$ MCF-7 cells in 200 μl phosphate-buffered saline. Tumors were measured using a caliper every 3 days. Animal experimentation was approved by the veterinary office of the Kanton Zürich (approval number 04/2008).

Tissue specimen and TMA analysis
For immunohistochemistry, our study included TMAs of 281 invasive breast cancer cases diagnosed at the Institute of Surgical Pathology (University Hospital, Zürich, Switzerland) as described (Theurillat *et al*., 2007). Tumor histology was determined according to the criteria of the World Health Organization (2003), whereas disease stage was assessed according to the International Union Against Cancer (UICC, 2002). Tumors were graded according to Bloom and Richardson, as modified by Elston and Ellis (1993) The clinicopathological characteristics of the patients/tumors are described in Supplementary Table 1. Patient age at the time of diagnosis ranged from 26 to 98 years with a median of 61 years (mean 62). For statistical analysis, only cases with clinical follow-up data were considered. The median observation time for overall survival was 61 months for patients still alive at the time of analysis. In all, 74 patients (27%) died during follow-up. Data on adjuvant therapy was not available. PHD2 expression was semiquantitatively scored as low or high.

Statistical analysis
Where not otherwise indicated, results are presented as mean values ± standard error of the mean of at least $n=3$ independent experiments. *P*-values were obtained by unpaired *t*-tests ($*P<0.05$, $**P<0.01$, $***P<0.001$). For statistical TMA analysis, PHD2 expression data were analyzed in crosstables with χ^2-test. Survival analysis was conducted according to Kaplan–Meier with log rank test and multivariate Cox regression analysis (inclusion model). All statistics were calculated using PASW (SPSS 18) software.

Immunohistochemistry
Dissected tumors were macroscopically evaluated, cut in half, and frozen in embedding medium (Tissue-Tek OCT compound, Sakura Finetek, Staufen, Germany). Acetone-fixed cryosections (8 μm) were blocked with 0.5% bovine serum albumin and incubated with anti-PECAM-1 antibody (BD Biosciences) for 1 h at room temperature to stain blood vessels. Tumor sections were further incubated with Alexa 568-conjugated goat anti-rat antibodies (Invitrogen) followed by nuclear staining with 4,6-diamidino-2-phenylindole and mounting in Prolong medium (Invitrogen). Blood vessel density, area and size were quantified using Axio Vision software (Carl Zeiss, Feldbach, Switzerland). TMA sections were processed using an automated immunohistochemistry platform (Benchmark, Ventana, Roche, Illkirch, France) with a PHD2 polyclonal anti-PHD2 antibody at 1:100 dilution (NB100-137; Novus Biologicals).

shRNA constructs and lentiviral infections
Expression vectors encoding shRNA sequences targeting human PHD2, HIF-1α, HIF-2α and amphiregulin driven by the U6 promoter in a pLKO.1-puro plasmid were purchased from Sigma. Variants of shRNA expression plasmids bearing a hygromycin resistance marker were generated by replacing the original puromycin resistance cassette in pLKO.1. Viral particles were produced in HEK293T cells using the ViraPower lentiviral expression system according to the manufacturer's instructions (Invitrogen).

RNA interference
MCF-7 cells were transfected with 100 nM siRNA duplex oligonucleotides using Lipofectamine 2000 (Invitrogen). The following RNA interference sequences (Invitrogen) were used: control siRNA, forward 5′-GCUCCGGAGAACUACCAG AGUAUUA-3′; HIF-2α siRNA, forward 5′-CAGGUGGA GCUAACAGGACAUAGUA-3′; RelA siRNA, forward 5′-AUCCGGUGACGAUCGUCUGUAUCUG-3′.

Proteome profiler
Cells (1.2×10^6) were incubated for 48 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Supernatants were harvested and treated according to manufacturer's protocol (Human Angiogenesis Array kit, R&D Systems Europe, Abingdon, UK). Proteome profiler arrays were carried out in triplicates and data are presented as ratios relative to the positive controls.

Tube formation assay
Cells (1×10^5) were incubated for 48 h in endothelial basal medium-2 medium containing 2% fetal calf serum and supernatants were harvested. HUVEC cells (7.5×10^3) were resuspended in 50 μl conditioned medium and plated into the wells of Angiogenesis μ-slides (Ibidi, Vitaris, Baar, Switzerland) coated with 3 mg/ml Matrigel (BD Biosciences). Tube formation was photographed after 6 h. Quantification was performed either by counting the branching points, defined as a vessel that subdivides in at least two additional vessels, or by measuring the length of the tubes using Axio Vision software (Carl Zeiss).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Supplementary Figure S1

PHD2 protein levels were determined in parental and shRNA control MCF-7 cells by immunoblotting. Cells were cultivated under hypoxic conditions (1% O_2) for up to 72 hours.

Supplementary Figure S2

Cell proliferation of parental MCF-7 cells was assessed by counting cells over 11 days. Cells were treated with either 2 nM estradiol or 1 □M of the ER antagonist fulvestrant. DMSO was used as solvent control.

Supplementary Figure S3

Transcript and protein levels of PHD2 in parental, shPHD2 C1 and B1 tumors were measured by RT-qPCR (upper panel) and immunoblotting (lower panel), respectively.

Supplementary Figure S4

PHD2 immunohistochemistry of parental and shPHD2 C1 MCF-7 cells.

Supplementary Figure S5

Protein levels of PHD2 were determined in pool of clones of lentivirally transfected shPHD2 and sh-control MCF-7 cells by immunoblotting. Cells were cultured under normoxic (20% O_2) or hypoxic (1% O_2) conditions for 24 hours.

Supplementary Figure S6

Membranes containing arrays of antibodies derived against human angiogenic factors were incubated with supernatants from parental, shPHD2 and PHD2-reconstituted MCF-7 cells. Quantification of membrane data in lower panels are presented as pixel densities relative to the positive controls.

Supplementary Figure S7

Transcript levels of amphiregulin were measured by RT-qPCR in parental and sh amphiregulin MCF-7 cells.

Supplementary Figure S8

MCF-7 cells were cultured under normoxic conditions in the absence or presence of 2 mM DMOG for up to 240 minutes. HIF-1□ and PHD2 protein levels were determined by immunoblotting of total cell lysates (upper panel). Transcript levels of amphiregulin, PHD2 and CA9 were determined by RT-qPCR (lower panel).

Supplementary Figure S9

Amphiregulin and TNF□ mRNA levels were measured by RT-qPCR in parental MCF-7 cells treated without or with 20 ng/ml TNF□ for the time periods indicated.

Supplementary Figure S10

Transcript levels of RelA, amphiregulin, I□B□, TNF□ and CA9 were analyzed in shPHD2 MCF-7 cells without or with (hatched columns) siRelA by RT-qPCR.

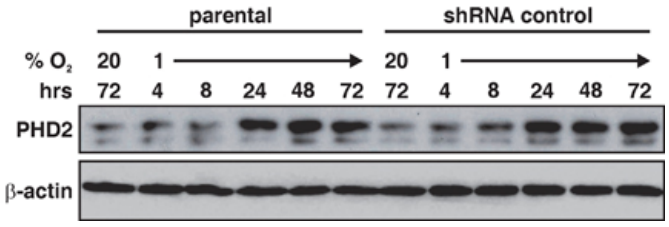
Supplementary Figure S11

Schematic summary of PHD2- and HIF-2□-dependent amphiregulin regulation. Besides the classical oxygen-dependent and 2-oxoglutarate (2-OG)-dependent signaling pathways, regulating e.g. VEGF, PHD2 might have additional functions as controling amphiregulin transcription indirectly or directly involving HIF-2□ and other cellular factors.

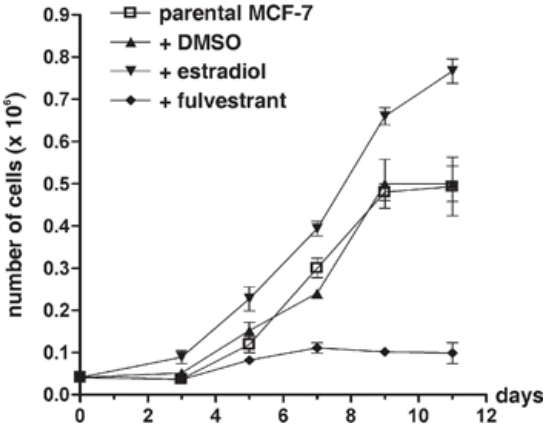
Supplementary Table 1

Clinico-pathological parameters of invasive breast cancer cases and relation to PHD2 expression.

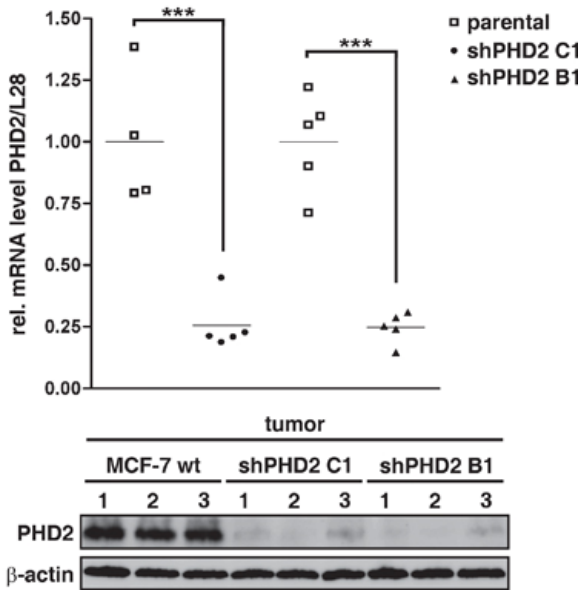
Supplementary Figure S1, Bordoli et al.



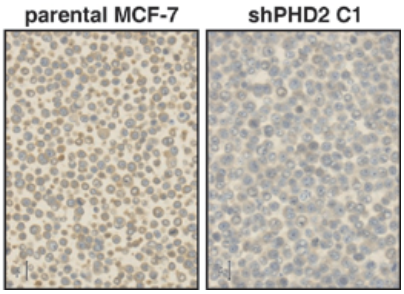
Supplementary Figure S2, Bordoli et al.



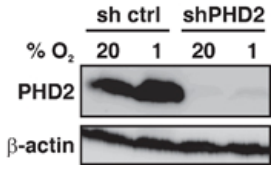
Supplementary Figure S3, Bordoli et al.



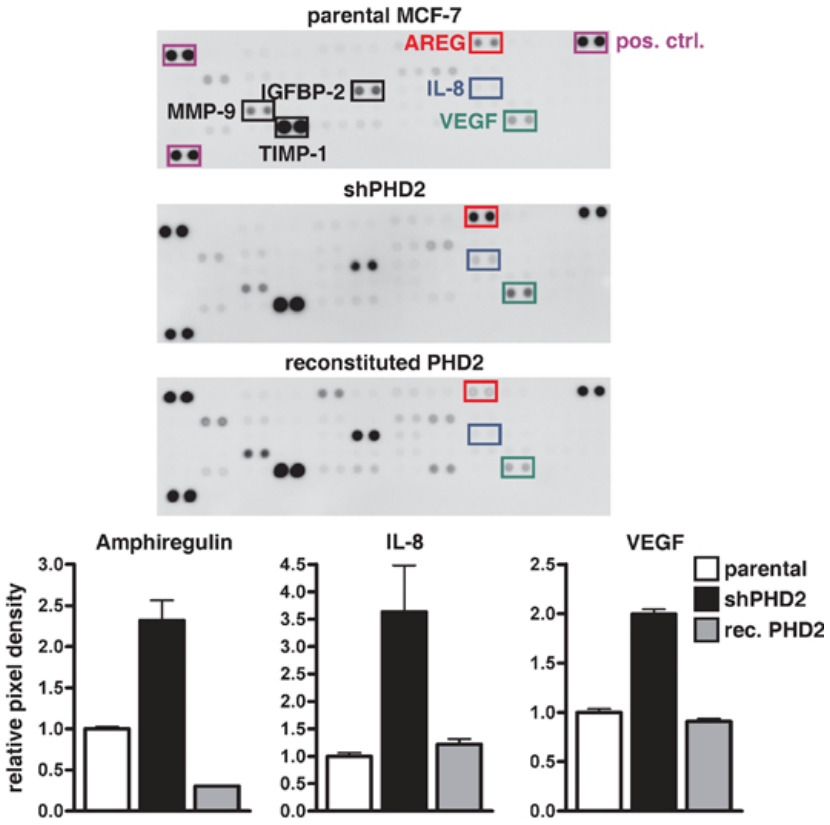
Supplementary Figure S4, Bordoli et al.



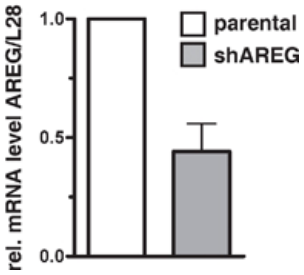
Supplementary Figure S5, Bordoli et al.



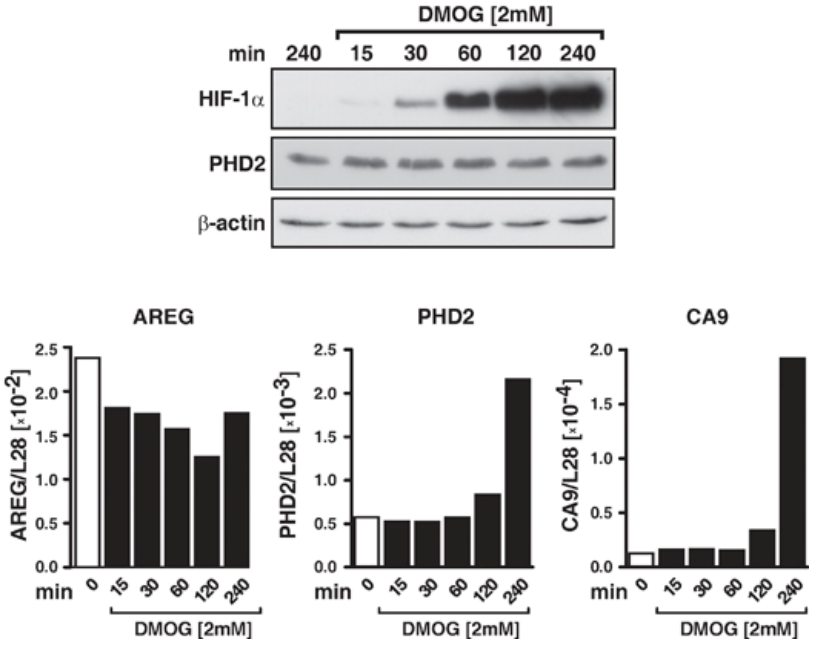
Supplementary Figure S6, Bordoli et al.



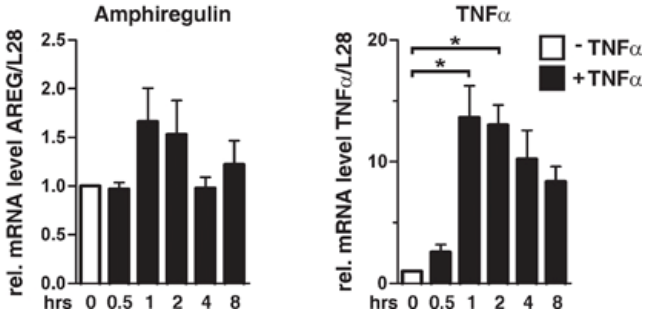
Supplementary Figure S7, Bordoli et al.



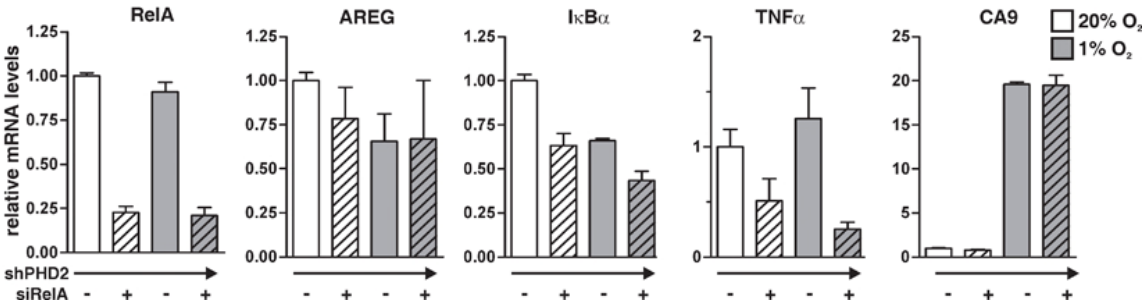
Supplementary Figure S8, Bordoli et al.



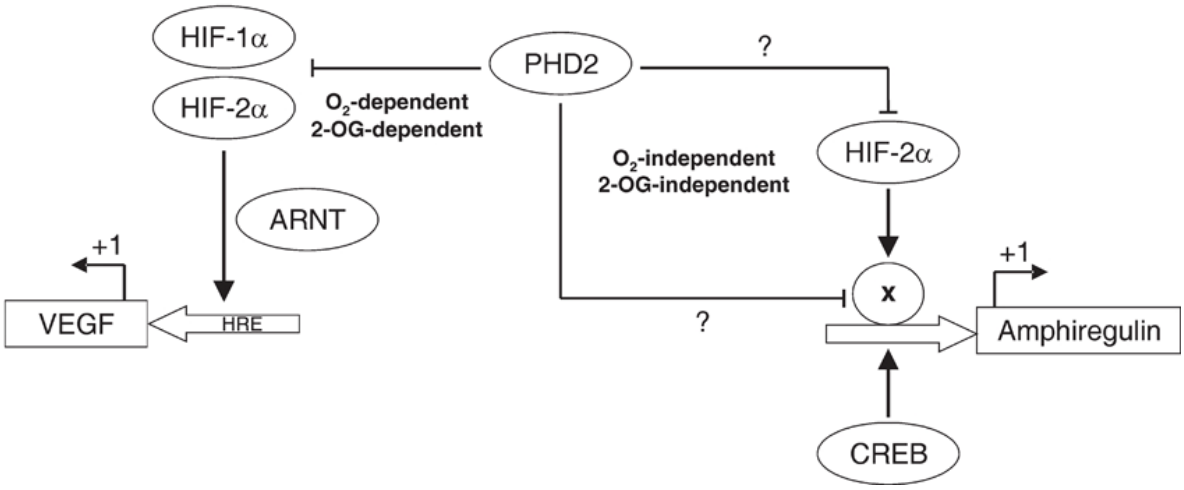
Supplementary Figure S9, Bordoli et al.



Supplementary Figure S10, Bordoli et al.



Supplementary Figure S11, Bordoli et al.



Supplementary Table 1

Characteristic	Number of cases (%)	PHD2 low	PHD2 high	*P*-Value
	273 (100%)	82	191	
< 60 years	119 (43.6%)	32	87	0.320[#]
>= 60 years	154 (56.4%)	50	104	
Pre-menopausal	55 (20.1%)	9	46	0.013[#]
Post-menopausal	218 (79.9%)	73	145	
Invasive ductal	221 (81.0%)	65	157	0.563*
Invasive lobular	39 (14.3%)	12	27	
NOS	18 (4.7%)	6	7	
pT1	92 (33.7%)	27	65	0.866[#]
pT2	122 (44.7%)	39	83	
pT3	15 (5.5%)	3	12	
pT4	44 (16.1%)	13	31	
pN0	103 (44.0%)	28	75	0.486[#]
pN1	106 (45.3%)	33	73	
pN2	17 (7.3%)	5	12	
pN3	8 (3.4%)	3	5	
G1	49 (17.9%)	18	31	0.329[#]
G2	138 (50.5%)	40	98	
G3	86 (31.5%)	24	62	
ER-negative	45 (17.7%)	10	35	0.378[#]
ER-positive	209 (82.3%)	60	149	
PR-negative	94 (34.7%)	28	66	0.979[#]
PR-positive	177 (65.3%)	53	124	
HER2 0, 1+, 2+	236 (90.4%)	74	162	0.111[#]
HER2 3+	25 (9.6%)	4	21	

[#]Chi-Square for trends, *Pearson Chi-Square

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DP Stiehl, **MR Bordoli**, I Abreu-Rodriguez, P Schraml, K Wollenick, G Kristiansen, RH Wenger, **HIF-2α dependent regulation of autonomous cell growth by the AREG-EGFR-ErbB4 axis is associated with significantly improved patient survival in human breast cancer** *(submitted)*

HIF-2α dependent regulation of autonomous cell growth by the AREG-EGFR-ErbB4 axis is associated with significantly improved patient survival in human breast cancer

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Running Title:
Distinct roles for HIF-α isoforms in breast cancer

Authorship note: GK and RHW contributed equally to this study

Summary

Hypoxia-inducible factor (HIF) 1 and 2 master the transcriptional response to local tissue hypoxia, a hallmark of solid cancers. Here, we report significantly longer patient survival in breast cancer with high levels of HIF-2α. Amphiregulin (AREG) and WISP2 expression was strongly HIF-2α dependent . The *AREG* promoter was strictly responsive to HIF-2α but independent of a classical HIF-DNA interaction motif, revealing a novel mechanism of gene regulation. Loss of AREG expression in HIF-2α depleted cells was accompanied by reduced activation of EGF-receptor family members and decreased hypoxic survival of tumor cells. Our results support a model in which breast cancer progression is dampened by a high HIF-2α/HIF-1α ratio, involving autocrine action of HIF-2 dependent growth factor secretion.

Significance

Tumor progression is intrinsically tied to clonal selection of tumor cells with acquired phenotypes allowing to cope with a hostile microenvironment. In practice, cytotoxic therapies may further enhance this selection pressure, often leading to relapse of the disease after an initial phase of tumor mass reduction. Our findings imply that HIF-2α mediated autocrine growth signaling in breast cancer sustains a state of cellular autarchy, thereby masking unfavourable growth conditions. Importantly, HIF-2α/AREG/WISP2 expressing tumors were associated with luminal tumor differentiation, indicative for a better response to classical treatment. In summary, shifting the HIF-α balance towards an HIF-2 dominated phenotype could offer a novel approach in breast cancer therapy

Introduction

A mismatch between metabolic demand and oxygen delivery leads to locally occurring hypoxia and cellular energy crisis in solid tumors. Hypoxia-inducible factors (HIFs) mediate the transcriptional adaptation of hypoxic cells to these microenvironmental conditions (Bertout et al., 2008). HIFs are composed of constitutively expressed β-subunits and one out of two oxygen-labile α-isoforms, resulting in transcriptionally active HIF-1 and HIF-2 heterodimers, respectively. Though structurally highly similar, specific roles of HIF-α isoforms in tumorigenesis have been defined (Patel and Simon, 2008; Semenza, 2010). Interestingly, ongoing hypoxia in a variety of human cancer cell lines leads to a specific downregulation of HIF-1α while HIF-2α protein levels rather increase, suggesting adaptive activation of the hypoxia-responsive pathway during acute and chronic tissue hypoxia (Holmquist-Mengelbier et al., 2006; Stiehl et al., 2006). A transient activation of HIF-1 could also be observed in growing tumors *in vivo* using an allografted murine tumor model (Lehmann et al., 2009). As a consequence of the low structural order of neoplastic tissues and given the enormous plasticity of the tumor sustaining vasculature, spatial and temporal tumor oxygenation is highly variable (Dewhirst et al., 2008; Lehmann et al., 2009). Thus, we speculated that acute temporal fluctuations in tumor oxygenation preferentially induced HIF-1, while chronically hypoxic tumor areas would be marked by enhanced HIF-2 levels. To study the transcriptional ramifications of distinct accumulation tendencies of HIF-αs under acutely and chronically hypoxic conditions, we stably downregulated HIF-1α and/or HIF-2α by short hairpin RNA interference in a cellular model of hormone-sensitive breast cancer.

HIF-α isoform specific expression profiling by quantitative PCR revealed 6 groups of target genes with highly similar temporal transcription profiles. Target gene specificity was indeed marked by phaseal expression patterns, indicating HIF-1 as the dominant effector of acute responses to oxygen deprivation , while HIF-2 activity was preferentially active at sustained hypoxia and particularly in the absence of HIF-1. These cellular mRNA data were further correlated with protein levels in 282 breast cancer samples and associated with patient survival times.

Results

HIF-1α dominates the acute response to hypoxia by suppressing *HIF2A* gene expression

As described earlier (Stiehl et al., 2006), HIF-1α protein levels in parental MCF-7 cells cultured for up to 72 hours at 1% oxygen where maximally induced after 4 hours of hypoxic exposure and constantly declined during prolonged hypoxia, while HIF-2α levels showed delayed induction kinetics with peak levels observed after 24-48 hours (Figures 1A-C). Surprisingly, cell lines depleted of HIF-1α (shHIF1A #16, 21 and 24, respectively) had strikingly increased HIF-2α levels compared to the parental cell pool and HIF-2α protein was steadily rising under chronic hypoxia (Figure 1A). A similar increase in HIF-2α transcript levels under prolonged hypoxia was observed only in the absence of HIF-1α (Figure 1D, lower panel), indicating a model of mutual exclusion of both isoforms with a dominant role of HIF-1α, suppressing *HIF2A/EPAS1* gene expression. In line with such a hierarchical model, stable knock-down of HIF-2α expression in three independent cell pools (shHIF2A #1, 4 and 5) had only minor effects on the remaining HIF-1α isoform, with slightly increased protein levels under prolonged hypoxia (Figure 1B). The drastic drop in HIF-1α transcript levels observed in response to acute hypoxia (8 hours) did not affect protein levels at the same sampling time (Figures 1B and D), suggesting at least partially independent regulation on the mRNA and protein levels.

Among the HIF target genes, hypoxically induced HIF prolyl-4-hydroxylases PHD2 and PHD3 as well as the regulator of protein translation REDD1 have been implicated in regulating HIF-α protein expression under hypoxic conditions (Brugarolas et al., 2004; Minamishima et al., 2009; Stiehl et al., 2006). While basal levels of PHD2 were unaffected in both HIF-α knock-down models, the hypoxic induction of PHD2 was abolished specifically in the absence of HIF-1α Figures 1A and B). Notably, knock-down efficiency for all HIF-α cell lines and pools was >80% and HIF-1α knock-down proved sufficient to abrogate expression of carbonic anhydrase 9 (CA9), another well characterized HIF target gene (Wykoff et al., 2000) (Figures 1A and D). Both, PHD3 and REDD1 hypoxic expression kinetics were markedly delayed in the absence of HIF-1α, but only PHD3 showed 2 to 3 fold increased hypoxic protein accumulation in shHIF2A cell pools (Figures 1A and B).

To test if residual hypoxic induction of PHD3 and REDD1 was caused by redundancy of HIF-α isoforms, a double-knock down of both isoforms was generated. As shown in Figure 1C, hypoxic induction of PHD3 was fully lost in shH1A/H2A double knock-down pools and REDD1 was only marginally induced by hypoxia, confirming that the delayed hypoxic response observed for both proteins in shHIF1A clones indeed was mediated by HIF-2.

HIF-α isoforms determine target gene kinetics rather than specificity

Prompted by the variability of the hypoxic response and target-specificity of HIF-α isoforms, we were interested in testing whether our model cell lines would prove useful to identify novel profiles of co-regulated genes in a setting of acute and chronic hypoxia. Temporal gene expression patterns of 34 known HIF target genes (Aprelikova et al., 2006; Elvidge et al., 2006) were analyzed for HIF-1 and HIF-2 dependency by quantitative PCR in cells exposed to 1% oxygen for 4-72 hours. Hierarchical clustering of the samples identified a delay of hypoxic target gene expression in HIF-1α knock-down cells as the main feature caused by loss of this HIF-α isoform, while chronically hypoxic samples with HIF-1α knock-down clustered close to the respective samples in parental MCF-7 (Figure 2A). In contrast, all hypoxic timepoints from HIF-2α depleted cells clearly clustered from parental and shHIF1A knock-down MCF-7, indicating that HIF-2α is more than a “tardy” substitute for HIF-1α.

Unbiased grouping of putatively related hypoxic gene expression profiles applying *k*-means clustering resulted in 6 distinct groups with 2-9 set members (Figure 2B). The mean hypoxic expression profiles from all members in one group showed surprisingly low variability, implying the existence of mutual regulatory mechanisms of hypoxic gene activation (see red curves in Figure 2C representing $\log_2$ ratios of relative gene expression levels). As reported previously, the large majority of genes (e.g. glycolytic enzymes and genes involved in metabolic adaptation to low oxygen availability) were predominantly depending on HIF-1α (Hu et al., 2003; Iyer et al., 1998; Semenza et al., 1994). Nevertheless, our kinetic profiling revealed subtle differences in HIF-α dependency, resulting in a novel grouping irrespective of known gene functions. Group 1 contains the two genes with highest hypoxic induction factors, and essentially required HIF-1α for gene transcription, highlighting their putative value as hypoxia marker genes. Group 2

encompassed target genes such as PHD3 with delayed hypoxic induction in the absence of HIF-1α and complete loss of hypoxic induction when both HIF-α isoforms were ablated, suggesting that these genes are required in both acute and chronic hypoxia. Group 6 included genes with a highly similar regulation in response to HIF-α isoforms, but differed from group 2 by a transient expression profile in parental MCF-7, categorizing these genes as markers of acute hypoxia. Genes in group 4 were also transiently upregulated by hypoxia in parental MCF-7, but fully lost hypoxic induction when HIF-1α was missing. Rather unexpectedly, group 5 genes, including classical HIF target genes such as vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT1), were only marginally affected by depletion of one HIF-α isoform and were robustly induced by hypoxia even when both HIF-α isoforms were suppressed. Of note, HIF-1α has been found to form kinetically stable complexes with the native VEGF promoter with a half-life of more than 1 hour (Yu and Kodadek, 2007). While it is unclear, how these findings relate to the dynamics of promoter-bound HIFs in general, one might suspect that certain promoter environments stabilize DNA-bound HIFs more efficiently than others and thus still strongly respond to hypoxia if only low levels of HIFs are present.

Only few genes were identified which were selectively activated by HIF-2. All genes downregulated in the absence of HIF-2α were only modestly induced by hypoxia in parental cells but were strikingly super-induced at late time points in the absence of HIF-1α. Since this observation could reflect the robust hypoxic induction of HIF-2α mRNA and protein in shHIF1A cell lines, we re-analysed the data with specific focus on genes with highest expression levels under these conditions: Surprisingly, mRNA levels of IGFBP3, LOXL2 and EGFR followed a similar expression profile with pronounced hypoxic induction in shHIF1A cells, though transcript levels of these genes were only mildly affected by knock down of HIF-2α (Figure S1). Obviously, either HIF-α isoform can induce this set of genes, while HIF-2α seems to be a particularly potent activator.

HIF-2α/AREG/WISP2 defines a positive prognostic axis in human breast cancer

To study the selective role of HIF-α isoforms in breast tumorigenesis, tissue-microarrays (TMAs) of tumor samples derived from 282 breast cancer patients with

primary mamma carcinoma were stained for HIF-1α and HIF-2α. Antibodies used for immunohistochemical staining of paraffine-embedded tissue sections were validated for target specificity on a test array composed of equally fixed and embedded cell pellets from respective HIF-α knock-down cell lines (Figure S2). While overall survival of patient cohorts with low versus high nuclear expression of HIF-1α in neoplastic cells did not differ, patients with HIF-2α expressing tumors lived significantly longer ($p<0.01$, Log rank test) than those with tumors expressing little or no HIF-2α (Figure 3A˜) In line with this observation, HIF-2α expression levels in tumors correlated with low tumor grading ($p<0.01$, Spearman's *rho*), while in cases with high expression levels of HIF-1α a significant association ($p<0.01$, Spearman's *rho*) with disseminated decay of lymph nodes was observed (Figure 3B).

Given the low number of selectively HIF-2α regulated target genes found in MCF-7 cells, TMAs were further stained for inositol-1,4,5-triphosphate receptor type 1 (ITPR1), plasminogen activator inhibitor-1 (PAI1), Wnt-1-inducible signaling pathway protein-2 (WISP2) and amphiregulin (AREG), an epidermal growth factor (EGF) family member which we recently identified as dramatically downregulated in MCF-7 cells lacking HIF-2α (Bordoli et al., 2010). Immunohistochemical analyses of CA9 and GLUT1 were included as established histological markers of tissue hypoxia (Tomes et al., 2003; Wykoff et al., 2000). Rank order correlations confirmed that AREG and WISP2 were significantly more abundant in tumors also positive for HIF-2α with correlation coefficients of 0.442 ($p<0.01$; $n=192$) and 0.264 ($p<0.01$; $n=192$), while PAI1 and CA9 showed no specific association with one particular HIF-α isoform, though both PAI1 and CA9 significantly correlated with HIF-α expression in general. No correlation was observed between ITPR1 and HIF-α expression levels. Uniquely amongst the proteins tested, GLUT1 and WISP2 levels reciprocally correlated with HIF-α isoforms, indicating that these factors might be useful to discriminate the dominant HIF-α isoform in breast cancers (Figure 3B, right panel).

Kaplan-Meier survival curves of patient subgroups whose tumors expressed both, AREG and WISP2 at high levels, revealed a significantly improved survival time ($p<0.01$; Log rank test) if compared to cohorts with expression of only one or neither of the growth factors (Figure 3C-D). To test for a putative relation between expression of HIF-2α, AREG and WISP2 we examined the co-aggregation of either parameter in patients where all three factors could be assessed ($n=192$). In the large

majority (76-84%) of cases aggregation with at least one other factor was observed and a substantial fraction (35-43%) was positive for all three features. Remarkably, exclusive expression of either HIF-2α, AREG or WISP2 alone was rare and ranged from 9-16% of all cases only (Figure 3E).

The WISP2 and AREG promoters are predominantly activated by HIF-2α

Motivated by the clinical evidence for a relation between HIF-2α, AREG and WISP2 expression and the prospective value of all three proteins as prognostic beneficial factors, we set out to investigate the molecular mechanisms underlying co-regulation of both growth factors. While hypoxic regulation of endogenous WISP2 transcript levels at least partially involved also HIF-1α, AREG mRNA was non-responsive to oxygen deprivation and basal levels were strikingly downregulated in both shHIF2A and HIF-α double knock-down MCF-7 pools (Figure 4A). Of note, endogenous gene expression and HIF-α dependency was fully recapitulated when transfecting MCF-7 cells and respective knock-down derivatives with luciferase reporter gene constructs driven by the promoters of the human *WISP2* and *AREG* genes, indicative for transcriptional control rather than altered transcript stability (Figure 4B). Re-expression of shRNA-insensitive HIF-2α in MCF-7 shHIF2A led to a dose-dependent induction of *WISP2* and *AREG* promoter activities, while only minor or no such effects were observed when cells were transfected with equal amounts of a HIF-1α expression plasmid (Figure 4C).

The *AREG* 5'-UTR is essential for transcriptional activation by HIF-2α

We previously mapped the minimal HIF-2α responsive region of the human *AREG* promoter (Bordoli et al., 2010). Surprisingly, this region encompassing 120 nucleotides in both directions of the transcriptional start site (Figure 5A, upper panel) does not contain the consensus HIF binding site (HBS) 5'-RCGTG-3' (Wenger et al., 2005), although strong activation by forced expression of HIF-2α was observed (Figure 5A, lower panel). The luciferase reporter gene construct pGL(P2P) driven by the human *PHD2* promoter demonstrated functional activity of exogenous HIF-1α expression and served as hypoxic control. Deletion of a major part of the *AREG* 5'-UTR completely abolished the co-activating effect of HIF-2α overexpression while basal *AREG* promoter activity was retained, suggesting an essential role of the 5'-

UTR in regulating *AREG* transcription (Figure 5A). However, the isolated *AREG* 5'-UTR failed to confer HIF-2α responsiveness to the heterologous reporter gene construct pGL3prom, driven by the *SV40* minimal promoter (Figure 5B, upper panel), whereas the HIF-dependent enhancer element derived from the human *PHD2* promoter in the same context readily responded to hypoxia and HIF-1α over-expression (Figure 5B, lower panel).

HIF-2α mediated activation of the *AREG* promoter requires cooperative sites

Since HIF-2α regulation of the *AREG* promoter could not be attributed to the canonical HIF pathway (lack of hypoxic inducibility, absence of a functional HBS), phylogenetic foot-print analyses were applied to identify other conserved regulatory elements in the minimal HIF-2α responsive region. Genomic sequences of *AREG* orthologs derived from 15 mammalian species with a conserved TATA-box were subjected to multiple sequence alignments, highlighting four elements of at least 3 base pair length with sequence similarities of >90% (Figure 5C, black boxes). Confirming our approach, two of these sites comprised experimentally described functional Wilms tumor suppressor 1 (WT-1) and cAMP response element-binding protein (CREB) responsive elements (WRE and CRE, respectively) in the *AREG* promoter (Lee et al., 1999). In addition, conserved E-box (5'-CAGGTG-3') and T-box (5'-CACACCT-3') elements were identified, putatively serving as recognition interfaces for basic Helix-Loop-Helix (bHLH) and T-box DNA-binding domains.

Disruption of the WRE (ΔWRE) as well as two truncations of the *AREG* 5'-UTR (pAREG-ΔSacI and pAREG-ΔSmaI; Figure 5C) significantly attenuated the specific responsiveness to HIF-2α of the respective reporter constructs (Figure 5D). Although mutation of the CRE site (ΔCRE) reduced basal *AREG* promoter activity by 75% as expected (data not shown), induction by HIF-2α was fully preserved. Mutation of the putative E-box (ΔE-box) and T-box (ΔT-box) motifs neither affected the basal reporter activity nor the response to forced expression of HIF-α (Figure 5D).

Autonomous cell growth is impaired in cells lacking HIF-2α

To test if decreased expression of WISP2 and AREG affects mitogenic signaling in HIF-2α depleted cells, the phosphorylation status of 16 receptor tyrosine kinases (RTKs) was assessed in MCF-7 cells and HIF-α knock-down pools. As

shown in Figure 6A, EGF and ErbB4 receptors were hypo-phosphorylated in serum-starved shHIF2A pools (0.1% FCS for 20 hours) when compared to the parental line. Concomitantly, reciprocal activation of the EGF-R was observed in shHIF1A pools, possibly explained by increased HIF-2α activity in these cells. Besides basal activation of members of the EGF receptor family (EGF-R, ErbB3, ErbB4), none of the other screened RTKs was activated in MCF-7, emphasizing EGF signaling as a major mitogenic pathway in this cellular breast cancer model. Notably, stimulation of starved MCF-7 cells with 10% FCS exclusively activated the IGF1-R, suggesting autotrophic activation of the EGF pathway in this line (Figures S3A and S3B). Supporting this notion, stimulation of HIF-2α knock-down pools with recombinant AREG particularly activated EGF and ErbB4 receptors, which were both hypo-phosphorylated in the absence of HIF-2α (Figure 6B). No receptor activation was observed in serum-starved cells similarly stimulated with recombinant WISP2 (data not shown).

When compared to parental MCF-7, HIF-2α depleted cells showed strikingly retarded proliferation at low oxygen concentration and were highly susceptible to serum depletion as reflected by decreased cell numbers when cultured under reduced FCS concentrations. *Vice versa*, repression of HIF-1α increased cellular proliferation in the presence of high FCS concentrations only, presumably due to synergistic action of tumor-cell derived factors with serum components (Figure 6C). Interestingly, these effects were exclusively observed in cells exposed to combined oxygen and serum starvation, an environment frequently found in solid tumors. In summary, hypoxia tolerance of cells depleted for HIF-2α was greatly impaired even in the presence of serum-derived growth factors, possibly explained by reduced autonomous growth factor signaling in these cells.

HIF-α isoforms are reciprocally associated with luminal and basal-like breast cancer

Based on gene expression signatures, breast carcinomas have recently been classified into subgroups resembling their suggested origination from basal-like (estrogen receptor (ESR) negative) or ESR positive, luminal mammary epithelial cells (Perou et al., 2000). Similar transcriptional characteristics were obtained for more than 50 widely-used breast cancer cell lines, subsequently categorized according to their gene cluster segregation as basal A, basal B or luminal cell

models (Kao et al., 2009; Neve et al., 2006). Since *EGFR* has been established as a marker gene of basal epithelial cells (Nielsen et al., 2004), we wondered whether the AREG-EGFR/ErbB4 autocrine loop is associated with one particular subtype of breast carcinomas.

Expression data for HIF-α predictive genes identified in our study (*AREG*, *PAI1*, *CA9* and *GLUT1*) as well as EGF-R family members (*EGFR*, *ErbB2*, *ErbB3* and *ErbB4*) and respective luminal (*ESR1*) and basal (*KRT5*) marker genes were extracted from a dataset which previously identified 8,750 variably expressed genes from 50 breast cancer cell lines (Kao et al., 2009). Of note, *WISP2* was not amongst the genes listed. As shown in Figure 7A, *AREG* was highly expressed specifically in *ESR1* positive luminal cell types, co-segregating with high transcript levels of *ErbB3* and *ErbB4,* but with low expression of *EGFR*. A second cluster with high *AREG* transcript levels included cell lines with low expression of ESR1 derived from non-tumorigenic mammary tissue (MCF-10A, 184A1 and hTERT-HME1) and *EGFR* over-expressing SUM149 and SUM102 cell lines, both showing low levels of the other *ErbB* family member. As expected, *EGFR* expression was specifically high in basal-like cell types, possibly indicating a leading role of ErbB4 in mediating autocrine effects of AREG in luminal cancers.

A highly significant correlation ($p<0.01$, Spearman's *rho)* between HIF-2α/AREG/WISP2 and proteins indicating luminal epithelial differentiation (ESR and progesterone receptor (PGR), respectively) was confirmed by immunohistochemistry in our cohort of 346 breast cancer patients. Reciprocally, HIF-1α as well as PAI1 and GLUT1 were significantly associated with expression of the basal cytokeratin KRT5 ($p<0.01$-0.05), suggesting a dominant role of HIF-1α in basal-like tumors (Figure 7B). In line with a luminal signature, AREG and WISP2 were found to be highly expressed in luminal cells of healthy mammary tissue (Figure 7C). Retaining expression of these factors thus could indicate a higher degree of tumor cell differentiation, which is further supported by the fact that AREG and WISP2 negatively correlated with tumor grading (with Spearman's *rho* equalling -0.169; $p<0.01$ and -0.233; $p<0.01$, respectively).

Discussion

Pioneering work using genetically modified mouse embryonic fibroblast with targeted disruption of both *Hif1a* alleles suggested a predominant role of HIF-1α for regulating gene expression in response to hypoxia (Park et al., 2003), a finding that was confirmed for a limited number of classical HIF target genes in human cell lines using transient RNA interference against both HIF-α isoforms (Sowter et al., 2003). However, expression analyses in a renal clear cell carcinoma-derived cell line deficient for HIF-1α revealed preserved hypoxic induction of many known HIF target genes, indicating that effectively both HIF-α isoforms contribute to hypoxia-induced transcription (Hu et al., 2003). Domain swapping experiments between HIF-1α and HIF-2α provided experimental evidence for interchangeability of the DNA binding portion of either isoform and attributed gene selectivity to the N-terminal transactivation domains of HIF-α (Hu et al., 2007; Lau et al., 2007). Indeed, genome-wide promoter tiling arrays identified a common 5'-RCGTG-3' binding motif for both HIF-1 and HIF-2 which is identical to our previously published consensus core hypoxia response element (HRE) defined by >70 characterised HIF target genes (Mole et al., 2009; Wenger et al., 2005). Remarkably, almost all HIF-2 binding gene *loci* were also bound by HIF-1 with high stringency, while a substantial number of *loci* bound HIF-1 exclusively proposing that HRE-bound HIF-2 can be fully integrated into the classical pathway activated by HIF-1. Yet, 15% of the HIF-2 (and even 30% of HIF-1) binding regions identified in the same study did not contain any consensus 5'-RCGTG-3' motif (Mole et al., 2009), calling for alternative modes of HIF-DNA interaction.

The HIF-2α responsive AREG promoter described in this study represents an example of such an HRE-independent gene regulation. Target gene specificity in this case likely arises from additional *trans*-acting factors and/or *cis*-regulatory elements that cooperate specifically with HIF-2α. In a variety of HIF-2α selective promoters (including the WISP2 promoter) HREs have previously been reported to co-operate with ETS family transcription factor binding sites (Aprelikova et al., 2006; Elvert et al., 2003; Hu et al., 2007; Le Bras et al., 2007; Wang et al.), and HIF-2α has been shown to physically interact with ETS-1 and ELK-1 (Aprelikova et al., 2006; Elvert et al., 2003). Other HIF-2α regulated genes at least contained one consensus HRE (Covello et al., 2006; Yamashita et al., 2008; Yang et al., 2010), although the stringency with which the core HRE has been determined was

sometimes lowered to only half-sites of the 5'-RCGTG-3' motif (Saito et al., 2010). Notably, no ETS-binding 5'-GGA(A/T)-3' core motif is present in the minimal HIF-2α responsive *AREG* promoter region**,** suggesting the existence of a so far unrecognized pathway.

By phylogenetic and subsequent mutational analysis of the minimal *AREG* promoter we identified a conserved WRE, mediating HIF-2α specific activation of AREG expression in MCF-7 breast cancer cells. Previous work identified the WT1(-KTS) splice variant as a potent transcriptional activator binding to this particular site (Lee et al., 1999). We could not detect any expression of WT1 mRNA in MCF-7 cells which is in line with a previous study on expression and epigenetic regulation of WT1 in human breast cancers (Loeb et al., 2001). Thus, a so far undefined WRE binding activity is likely to mediate *AREG* expression in response to HIF-2α. We cannot conclude from our experiments whether this regulation requires direct binding or indirect recruitment of HIF-2α to the DNA. Alternatively, induction of another transcription factor by HIF-2 might be involved.

Evidence supporting a model of a shared HIF-2α/AREG/WISP2 pathway arises from clinical data of 346 breast cancer patients, where a strikingly good correlation of either growth factor specifically with HIF-2α was observed. Only patients with high levels of both AREG and WISP2 showed similarly prolonged survival times like the HIF-2α positive cohort, indicating a concerted action of these proteins. Contrary to our results, another recent study found HIF-2α associated with distant recurrence and poor outcome in invasive breast cancer (Helczynska et al., 2008). HIF-2α antibody quality has been validated in both studies and hence is unlikely to explain the discrepancy. However, Helczynska *et al.* analysed breast cancer specific survival whereas our stratified analyses considered overall survival. In agreement with our data showing significant correlation of HIF-1α expression with disseminated disease stages, conditional deletion of HIF-1α in the mammary epithelium of mice expressing the PyMT oncoprotein resulted in retarded tumor growth and decreased pulmonary metastasis (Liao et al., 2007).

HIF-2α has been discussed as a critical mediator of renal tumorigenesis by enhancing c-*Myc* activity *in vivo*, particularly in renal cell carcinoma (RCC) exclusively expressing this isoform, and overexpression of HIF-2α increased xenografted tumor growth (Gordan et al., 2007; Gordan et al., 2008; Raval et al.,

2005). However, we did not observe any changes in c-*Myc* dependent transcription in MCF-7 cells lacking HIF-1α (Figure S4) implying that HIF-2α/c-*Myc* interplay probably is tissue specific. Surprisingly, stabilized HIF-2α mutants promoted growth of xenografted RCC cell tumors and *Kras*^{G12D}-driven lung tumorigenesis in mice (Kim et al., 2009; Yan et al., 2007), while *Hif2a* deletion in the same lung cancer model likewise increased tumor burden (Mazumdar et al., 2010). Though contradictory at first sight, Mazumdar *et al.* propose an appealing model where forced expression of HIF-2α enhances tumor growth by activation of tumor promoting target genes, while genetic silencing of HIF-2α leads to loss of tumor suppressor genes also boosting tumorigenesis (Mazumdar et al., 2010).

What could be the mechanism behind such a dual role of transcriptional regulation by a single factor? Interestingly, we identified two distinct groups of HIF-2 regulated genes: one set was particularly upregulated by HIF-2α in the absence of HIF-1α but still responding to hypoxia in a HIF-1α dependent fashion, whereas another set was exclusively restricted to HIF-2α. Therefore, HIF-2α could act bidirectionally via a canonical HRE-mediated pathway overlapping with HIF-1α plus an HRE-independent HIF-2α specific mechanism.

Intriguingly, all genes that clustered in HIF-2α affected groups were either transmembrane (*ITPR1, EGFR*) and/or secreted proteins (*IGFBP3, LOXL2, PAI1, WISP2, AREG*), suggesting a novel role of HIF-2α in regulating intercellular communication. Supporting this hypothesis, EGF-R and ErbB4 receptor activation was significantly lower in the absence of HIF-2α, and exactly those two receptors were predominantly activated by AREG. While our work was in progress, another study reported similarly reduced EGF-R phosphorylation in human glioma, lung and colon carcinoma cell lines depleted of HIF-2α, postulating a widespread mechanism of HIF-2α mediated growth control in human cancers (Franovic et al., 2009).

To the best of our knowledge, AREG establishes the first direct link between HIF-2α dependent transcription and EGF-R family activation in human breast cancers. AREG co-segregated with marker genes predictive for a luminal subtype of breast cancer, characterized by low EGF-R expression levels (Perou et al., 2000). Thus, ErbB4 might be the prime target of HIF-2α/AREG dependent activation, as transcript levels of this EGF receptor family member were particularly high in cell lines simultaneously expressing AREG. Moreover, we found a strong correlation of

HIF-2α, AREG and WISP2 protein levels in tumor samples with luminal differentiation, providing evidence that the HIF-2α specific transcriptional pathway could play an important role in maintaining a non-invasive phenotype, as it has been reported for tumors expressing WISP2 before (Banerjee et al., 2008; Fritah et al., 2008).

Growth of MCF-7 cells depleted of HIF-2α was impaired under oxygen and nutrient limiting conditions *in vitro*, but a negative correlation of HIF-2α with tumor size and grade was observed in clinical samples. Such disparate proliferation characteristics of cells cultured *in vitro* or grown as xenografts *in vivo* have been described previously for A549 lung carcinoma cells with reduced levels of HIF-2α (Franovic et al., 2009; Mazumdar et al., 2010). Cancer cells interact with the surrounding microenvironment by secretion of a variety of growth factors, partially mediated by a HIF-2α specific transcriptional pathway. In normoxia, a balanced activation of canonical and HIF-2 specific pathways tightly controls energy homeostasis and cellular proliferation (Figure 8A). Tumor cells, however, encounter periods of severe oxygen starvation, ultimately selecting for phenotypes resistant to a hostile environment (Gatenby et al., 2007). Thus, cancer cells expressing high levels of HIF-2α (like those cells with suppressed HIF-1α in our study) benefit from increased autocrine and paracrine growth factor signaling, supporting cellular autonomy by masking unfavourable growth conditions. As a consequence, selection pressure in these cancers might be reduced and tumor progression is slowed down (Figure 8B). On the other hand, cells predominantly expressing HIF-1α recruit oxygen-independent energy pathways but may suffer from decreased mitogenic signaling due to loss of HIF-2α specific growth factors. Increased selection pressure forcefully promotes tumorigenesis, eventually leading to the acquisition of invasive properties and dissemination of the disease (Figure 8C). Based on the recently developed concepts of “Adaptive Therapy”, where a stable tumor burden of higher differentiated tumor cells is sustained to suppress growth of slow proliferating but therapy resistant phenotypes (Gatenby et al., 2009), novel therapies maintaining a HIF-2α dominated phenotype could prove of value to treat breast cancer.

Experimental Procedures

Cell culture and derivatives

Human breast cancer MCF-7 cells were cultured in DMEM (high glucose, Sigma) supplemented with 10% FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin (Invitrogen). Cell lines with stable HIF-1α suppression (shHIF1A #16, #21 and #24) were generated by transfection with a pLKO.1-puro vector expressing U6 promoter driven shRNA targeting nucleotides 1168-1188 of human *HIF1A* (NM_001530.x-1048s1c1, Sigma). Cell clones were derived by puromycin selection (2 µg/ml) and ring cloning. Hypoxia kinetics were carried out at 1% oxygen and 5% CO_2 in a gas-controlled glove box (InvivO2 400, Ruskinn Technologies).

shRNA constructs and lentiviral infections

Lentiviral expression vectors encoding three shRNA sequences targeting human *HIF2A* at nucleotides 878-898 (#1, NM_001430.x-517s1c1), 927-947 (#5, NM_001430.x-566s1c1) and 2055-2075 (#4, NM_001430.x-1694s1c1) in a pLKO.1-puro plasmid were purchased from Sigma. Variants of shRNA expression plasmids bearing a hygromycin resistance marker were generated by replacing the original puromycin resistance cassette in pLKO.1. Viral particles were produced in HEK293T cells by co-transfection of the respective transfer vector (3 µg) with the packaging plasmids pLP1 (4.2 µg), pLP2 (2 µg) and pVSV-G (2.8 µg, all from Invitrogen) using PEI transfection as described before (Stiehl et al., 2006). MCF-7 cells were infected with lentiviral-pseudotyped particles and cell pools were derived by puromycin (2 µg/ml) and/or hygromycin (400 µg/ml) selection.

Tissue specimen and TMA analysis

Histopathological and immunohistochemical analyses of tumor tissues from 282 invasive breast cancer cases diagnosed at the Institute of Surgical Pathology (University Hospital, Zürich, Switzerland) were performed essentially as described {Bordoli, 2010 #1272}. Patient age at time of diagnosis ranged from 26 to 93 years with a median of 61 years (mean 62). The median observation time for overall survival was 61 months for patients still alive at the time of analysis. In total, 74 patients (26.2%) died during follow-up. Detailed information on the clinicopathological characteristics of the patients/tumors are described in Table S1.

Antibodies used for immunohistochemistry are listed in the *Supplemental Experimental Procedures*.

Human phospho-RTK array

The phosphorylation status of 16 receptor tyrosine kinases (RTK) has been assessed in parallel using Proteome Profiler 96 (R&D Systems). Briefly, 1×10^6 MCF-7 cells or derivatives were grown at 0.1% FCS for 20 hours and stimulated with recombinant AREG (100 ng/ml; R&D Systems), WISP2 (100 ng/ml; Creative Biomart) or 10% FCS for up to 20 minutes. Cells were lysed immediately and protein concentration was determined by the Bradford method. Cellular protein (1 to 25 μg) was subjected to the provided two-site sandwich ELISA plate and the assay performed following the manufacturer's instructions. Signals were detected using a luminescent image analyser (LAS-4000, FUJIFILM) and spot intensities were quantified with the Quantity One software package (Biorad).

Supplemental Information

Supplemental Information including *Supplemental Experimental Procedures*, four figures and one table can be found online with this article.

Acknowledgments

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Figures

Figure 1. Reciprocal induction kinetics of HIF-α isoforms: early HIF-1α, late HIF-2α

(A) MCF-7 (parental) and respective shHIF1A clones (#16, 21 and 24) or three independent stable cell pools, lentivirally infected with different targeting sequences for HIF2A (B), were subjected to 1% oxygen for up to 72 hours and whole cell protein extracts were immunoblotted for the proteins indicated. (C) Combined knock-down of both HIF-α isoforms was achieved by infecting clone shHIF1A #24 with pLKO.1-hygro shHIF2A #4. Extracts were analysed as above. (D) Transcript levels of parental, shHIF1A, shHIF2A and shH1A/H2A cultures were quantified by RT-(q)PCR. Control cells ("C") were cultured at 20% oxygen, while hypoxic ("hyp") probes were sampled at the same time points as shown in immunoblots. All data are given as mean ± SEM of three independent experiments and normalized to ribosomal protein L28 mRNA levels. Highest hypoxic mRNA levels in the parental cell line was arbitrarily defined 1 in each independent experiment.

Figure 2. HIF target genes cluster with distinct hypoxic expression profiles

(A) Dendrogram depicting hierarchical clustering of the hypoxic response in parental MCF-7 and HIF-α single and double knock-down cells based on expression kinetics of 34 known HIF target genes. (B) Transcript levels determined by RT-(q) PCR for known HIF target genes in parental, shHIF1, shHIF2 and double knock-down shH1A/H2A MCF-7 cells exposed to 1% oxygen for up to 72 hours. A heat map illustrates time-resolved hypoxic expression kinetics for each gene with a dynamical range from -3 (lowest expression) to +3 (highest expression) in blue and red, respectively. Data represent $\log_2$ (ratios) of the mean of three independent experiments normalized to L28 mRNA levels. Genes were grouped by time-resolved gene expression applying *K*-means clustering. (C) Hypoxic gene expression profiles of each group given as mean induction ($\log_2$ ratios) of all group members (red line and right y-axes). Grey-shaded areas indicate the dynamic range of the hypoxic response in parental cells. A representative gene of each group is also shown (bar graphs relate to left y-axes, mean ± SEM).

Figure 3. HIF-2α is a positive prognostic factor in breast cancer

(A) Immunohistochemical detection of HIF-α isoforms in tissue micro arrays (TMAs) of 282 invasive breast cancer cases. Kaplan-Meier survival curves are shown for patients with low or high expression of either isoform. Log-rank tests (Mantel-Cox) were performed to compare the survival distributions of the two groups. Lower panels are representative immunostains of tumor tissue with low and high HIF-α expression. Arrowheads indicate exemplary cells with nuclear HIF-α staining. (B) Rank-order correlations (Spearman's rho) for HIF-α forms with clinico-pathological characteristics (table) and isoform pre-selective HIF target genes (right panel). Disease stage was assessed according to International Union Against Cancer (UICC, 2002). Tumors were graded according to the modified Bloom and Richardson system. Asterisks indicate statistical significance with *p<0.05 and **p<0.01. (C) Overall survival of patients with expression of either AREG or WISP2 in primary tumors ("one"), both factors together ("both") or absence of either factor ("none"). Statistical analyses were performed as described above. (D) Immunostainings of tumor tissues from six patients with low and high AREG or WISP2 expression, respectively. (E) Set diagram reflecting the relation between expression of HIF-2α, AREG and WISP2 in primary breast cancer. Only tumor samples where all three parameters could be accessed were included in the analyses (192 cases). Areas of sets are representative for the number of cases with respective attributes. Relative values indicate the set size in relation to the number of cases showing expression of at least one factor (128 cases).

Figure 4. HIF-2α dependent regulation of *WISP2* and *AREG* promoters

(A) Hypoxic expression profiles of *WISP2* and *AREG* in the presence and absence of HIF-α isoforms in MCF-7 cells. Transcript levels of normoxic cultures ("C") were compared to those cultured at 1% oxygen ("hyp") for 4, 8, 24, 48 and 72 hours. Data are given as mean ± SEM of 3 independent experiments. (B) HIF-2α affects *WISP2* and *AREG* promoter activities. MCF-7 parental cells, no target control shRNA ("pLKO.1-puro") and HIF-α knock-down pools were transfected with reporter constructs driven by the human *WISP2* or *AREG* promoters. Data (mean ± SEM, n=3) was normalized to normoxic expression levels of the human PHD2 promoter and differences in transfection efficiency were controlled by co-expression of *renilla* luciferase. (C) Overexpression of HIF-2α strongly activates the human *WISP2* and

AREG promoters. MCF-7 shHIF2A pools were co-transfected with either human *WISP2* (left panel) or *AREG* (right panel) reporter constructs and increasing amounts of HIF-α encoding expression plasmids (200, 400 and 800 ng DNA, respectively). The empty expression vector ("empty") served as negative control. Transfected cells were cultured for 24 hours at 20% or 1% oxygen and luciferase activity was determined.

Figure 5. Phylogenetic footprint analysis of the minimal HIF-2α responsive *AREG* promoter

(A) Scheme depicting the minimal HIF-2α responsive AREG reporter construct and a deletion mutant lacking the *AREG* 5'-UTR. Numbers are relative to the transcriptional start site (TSS). Reporter constructs (200 ng) were co-transfected with 800 ng of HIF-α expression plasmids or empty vector. The human *PHD2* promoter pGL(P2P) and the promoterless pGL2b served as controls. Luciferase activity was determined of cells cultured at 20% or 1% oxygen for 24 hours. (B) The isolated *AREG* 5'-UTR is not sufficient to mediate activation by HIF-2α. *SV40* minimal promoter driven luciferase fused to the *AREG* 5'-UTR was co-transfected with HIF-α expression plasmids or empty vector and luciferase activity was measured as described above. The UTR-lacking plasmid (pGL3prom) and the hypoxia-responsive reporter pSVL(P2P)95bp served as controls. (C) Sequence similarity of the *AREG* promoter in 15 different species harbouring a 100% conserved TATAAA box (lower panel). Conserved elements of at least 3 bp with >90% sequence similarity were mutated as indicated in the upper scheme of the reporter construct. (D) Mutated and 3'-truncated (Sac I and Sma I, respectively) *AREG* reporter constructs were co-transfected with HIF-α expression plasmids and empty vector as before. Data is shown as responsiveness to HIF-α isoforms normalized to the empty expression vector derived from three independent transfections analysed in triplicates. Asterisks indicate significant differences between HIF-1α and HIF-2α responsiveness of the respective constructs (Tukey's Multiple Comparison Test).

Figure 6. Basal EGF and ErbB4 receptor activation is attenuated in cells lacking HIF-2α

(A) Human Phospho-RTK Array of MCF-7 parental and HIF-α knock-down pools. Representative images of cultures grown at 0.1% FCS for 20 hours are shown. Phosphorylation levels of the EGF receptor family were quantified from at least three independent cultures. Asterisks indicate significant differences of the mean as determined by unpaired Stundent's t-tests (*p<0.05; ***p<0.001). (B) Recombinant AREG activates EGF and ErbB4 receptors in shHIF2A knock-down cells. MCF-7 shHIF2A cells were grown at 0.1% FCS for 20 hours and stimulated with recombinant AREG (100 ng/ml) for up to 20 minutes. Phosphorylation levels of EGF receptor family members were quantified from three independent experiments. Strongest phosphorylation levels in each experiment were set as 1.0 and data are given as mean ± SEM. (C) Combined oxygen and growth factor starvation in MCF-7 parental and respective HIF-α knock-down pools. Cells were cultured for 72 hours at 1% oxygen in the presence of 0.1-10% FCS. Control cultures were grown at 20% oxygen in media supplemented with 10% FCS. Cell proliferation was determined by MTT assay and data expressed as relative growth [in %] compared to cells cultured at control conditions (mean ± SEM)

Figure 7. HIF-2α/AREG/WISP2 are co-expressed in luminal breast cancers

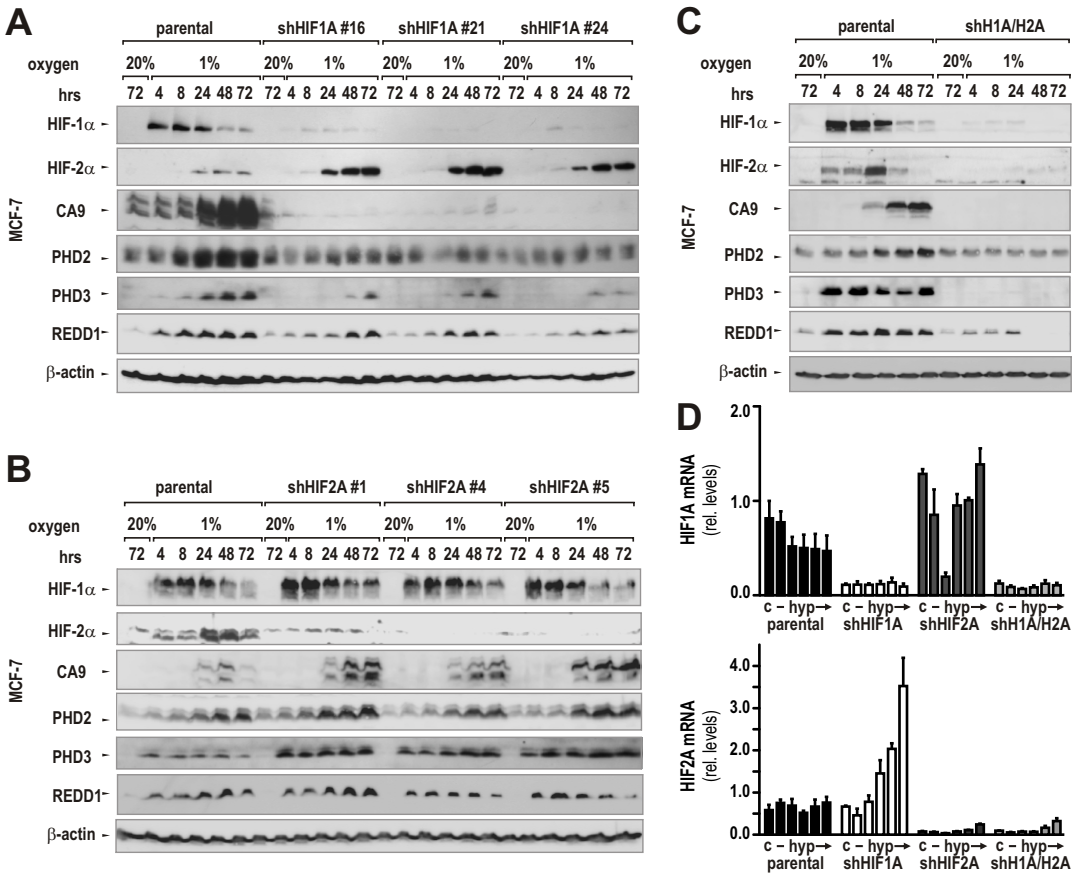
(A) Expression data for HIF-α predictive genes (*AREG, PAI1, CA9* and *GLUT1*) and EGF- receptor family members (*EGFR, ErbB2, ErbB3* and *ErbB4*) and respective luminal (*ESR1*) and basal (*KRT5*) marker genes in 50 breast cancer cell lines were extracted from (Kao et al., 2009). Hierarchical clustering (Euclidean distance) of expression values are depicted with a dynamical range from -3 (lowest expression) to +3 (highest expression) in blue and red, respectively. Cells have been characterized

previously as basal A (red), basal B (green) or luminal (blue) cell models according to cluster segregation with respective gene signatures (Kao et al., 2009; Neve et al., 2006). Black squares indicate missing data points. (B) Rank-order correlations (Spearman's rho) for protein expression of HIF-α isoforms and pre-selective HIF target genes with markers of luminal (ESR and PGR, left panel) as well as basal (KRT5 and EGF-R, right panel) cancer subtypes. Data were derived from immunohistochemistry of 346 tumor specimen for each protein. Asterisks indicate

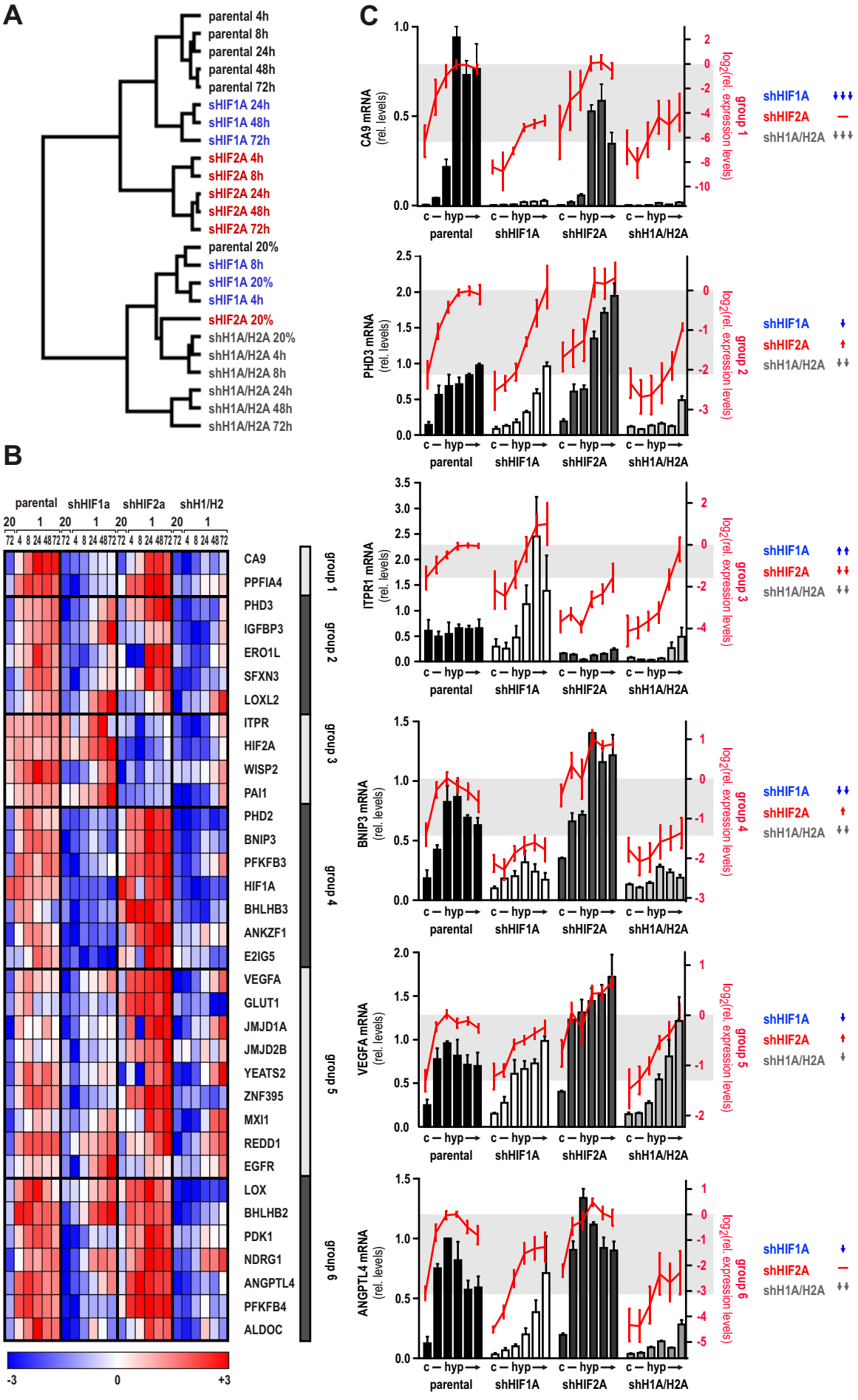
statistical significance with *p<0.05 and **p<0.01. (C) AREG and WISP2 are highly expressed in luminal cells in healthy breast tissue. Representative immunohistochemistry stains for AREG and WISP2 of a normal mammary lobule (left images). Arrowheads indicate luminal secretory and basal myoepithelial cell layers of healthy mammary epithelium (right images).

Figure 8. Autocrine growth signaling in breast tumorigenesis involves non-canonical functions of HIF-2α

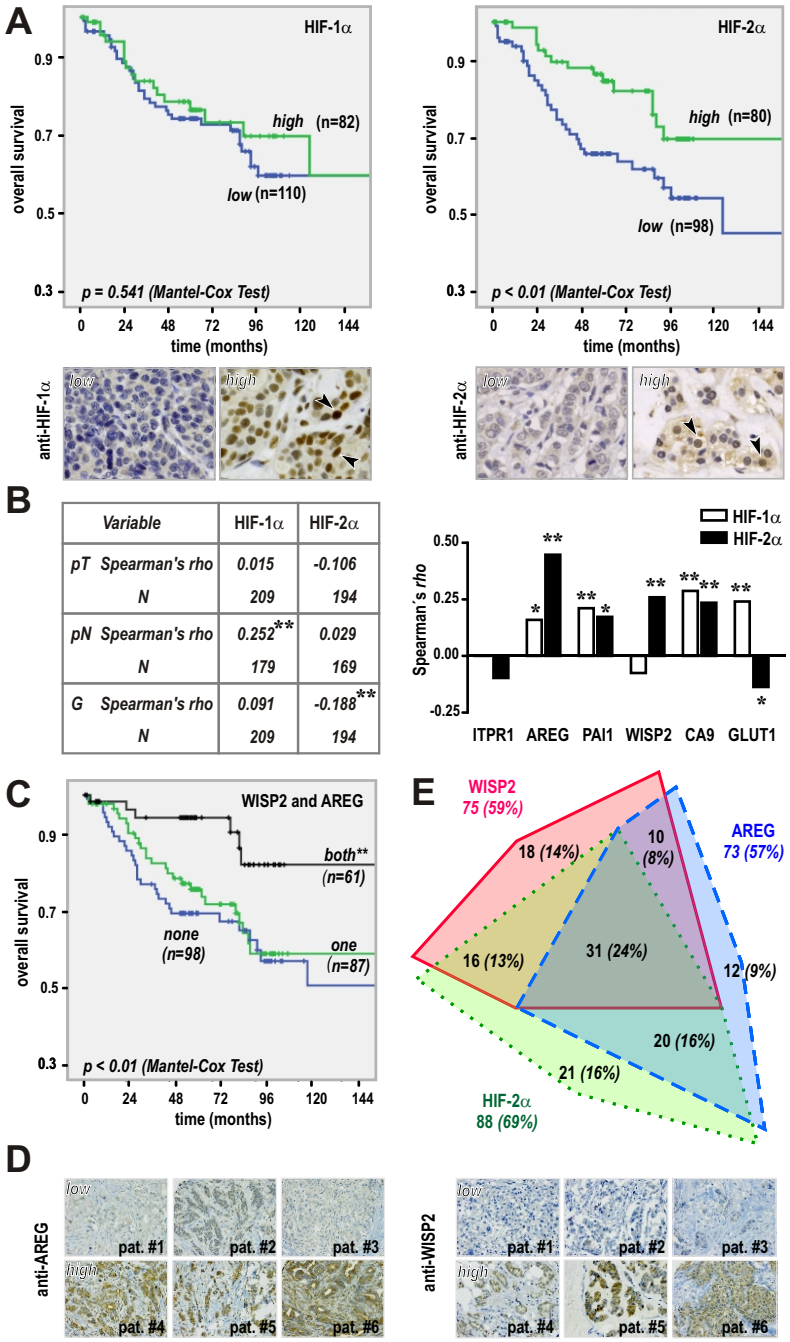
(A) At physiological oxygenation as found in developed tissues, the canonical (HRE-mediated) HIF pathway and HIF-2α specific signaling cooperatively regulate cellular energy metabolism and proliferation, respectively. HIF-α protein levels (left panel in all subfigures) reflect quantified band intensities (mean ± SEM) of immunoblots as presented in Figure 1. Highest protein levels observed in parental MCF-7 for either isoform were arbitrarily defined as 1. (B) Tumor cells overexpressing HIF-2α (simulated by knock-down of HIF-1α in our study) show increased activity of the autotrophic AREG/EGFR/ErbB4 pathway. *In vivo*, HIF-2α mediated cellular autonomy might slow down tumor evolution, e.g. by masking a hostile microenvironment. (C) Tumor cells with low expression of HIF-2α lack autotrophic growth signaling, increasing their addiction to microenvironmental growth stimuli. Starvation and a glycolytic phenotype boost tumor progression *in vivo*.



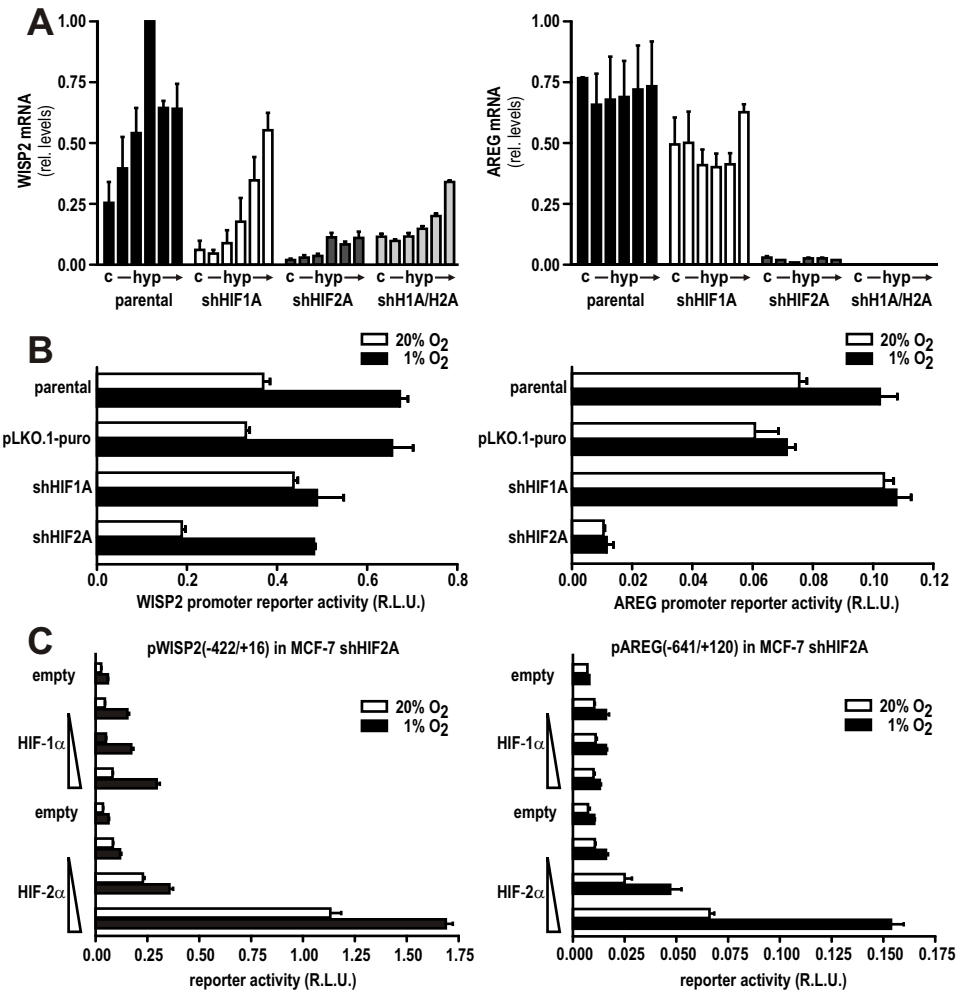
Stiehl et al. Figure 1



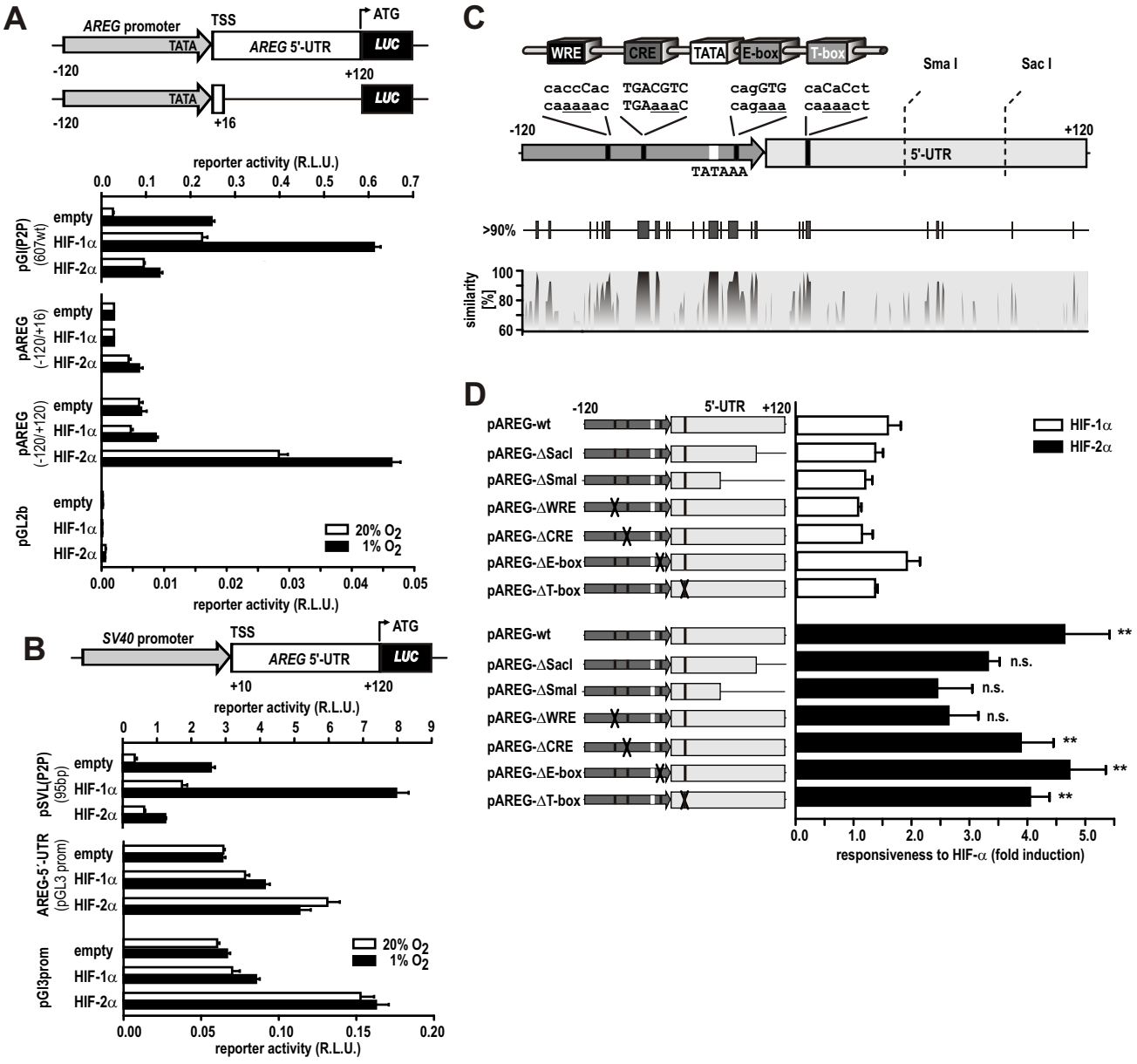
Stiehl et al. Figure 2



Stiehl et al. Figure 3



Stiehl et al. Figure 4



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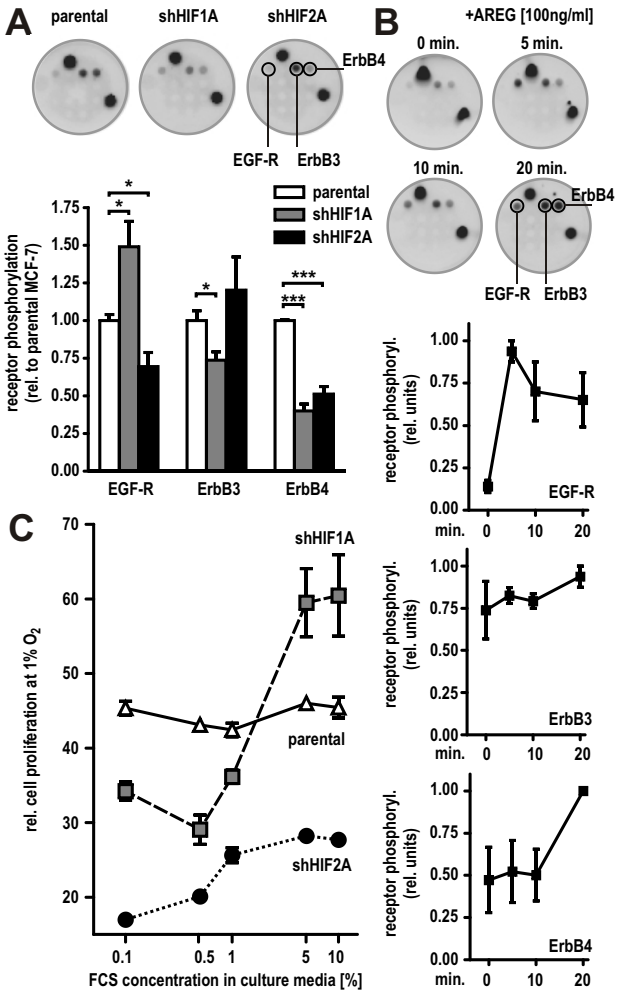
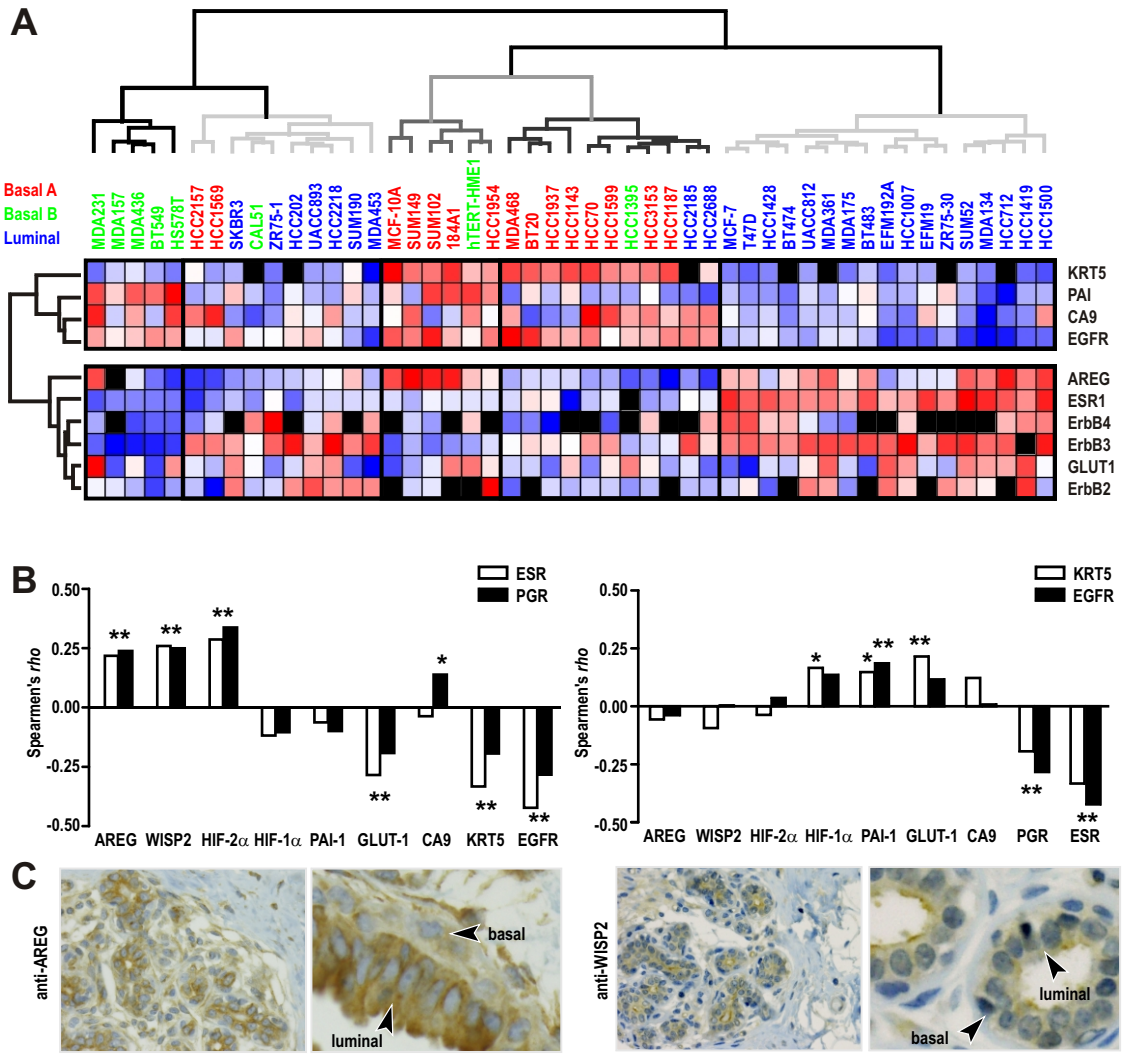
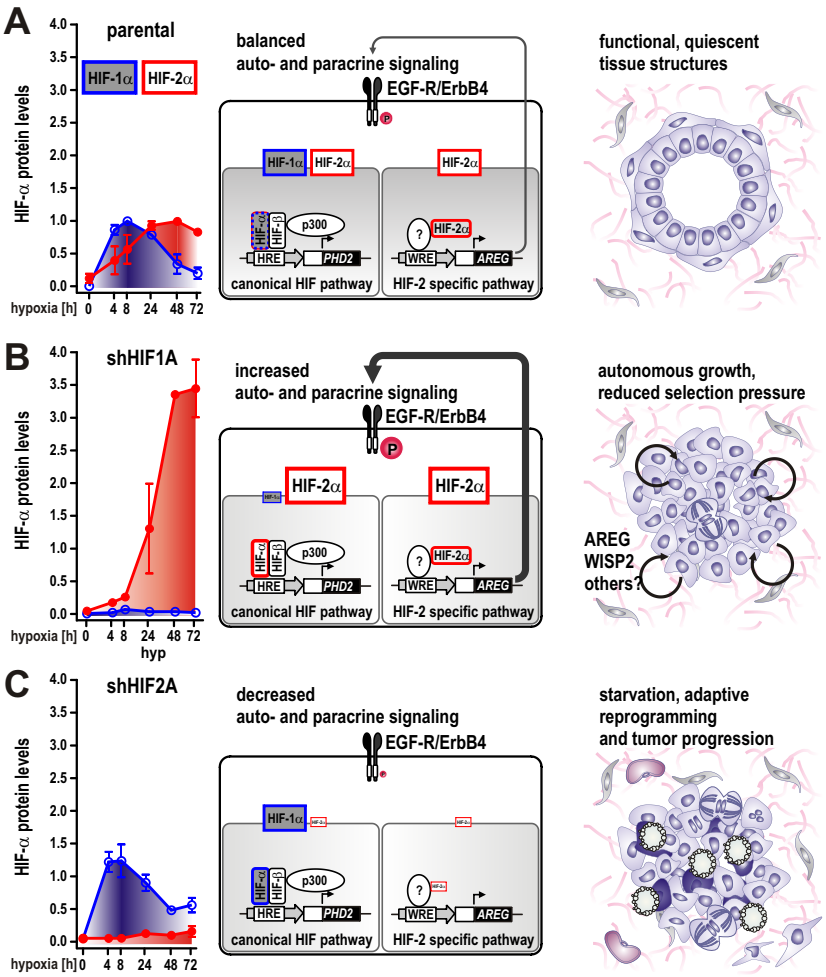


Stiehl et al. Figure 6



Stiehl et al. Figure 7



Stiehl et al. Figure 8

Supplemental Information

HIF-2α dependent regulation of autonomous cell growth by the AREG-EGFR-ErbB4 axis is associated with significantly improved patient survival in human breast cancer

Daniel P. Stiehl[1], Mattia R. Bordoli[1], Irene Abreu-Rodríguez[1], Kristin Wollenick[1], Peter Schraml[2], Glen Kristiansen[2] and Roland H. Wenger[1]

Inventory of Supplemental Information

Supplemental Data

Supplementary Figure 1 relates to Figure 2. Hypoxia-responsive genes strikingly upregulated in HIF-1α knock-down cells under chronic hypoxia

Supplementary Figure 2 relates to Figure 3. Validation of antibodies used for immunohistochemistry (IHC) on tissue microarrays (TMAs)

Supplementary Figure 3 relates to Figure 6. Autotrophic activation of EGF receptor family in MCF-7 breast carcinoma cells

Supplementary Figure 4 relates to Discussion. HIF-α knock-down only marginally affects c-*Myc* dependent gene expression in MCF-7 cells

Supplementary Table 1 relates to Figure 3. Clinicopathological parameters of invasive breast cancer cases and relation to HIF-α isoform expression.

Supplemental Experimental Procedures

mRNA quantification and primers
Plasmids and reporter gene assays
Immunoblotting
Cell proliferation/viability assays
Histopathologic grade and Immunohistochemistry
Bioinformatic analyses and statistical methods

Supplemental References

Supplemental Data

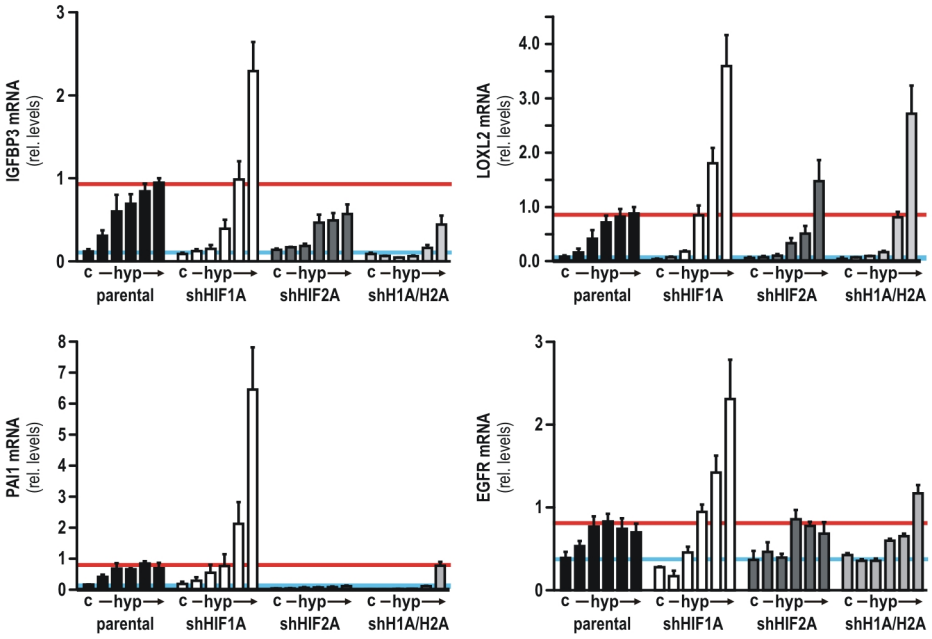


Figure S1. Hypoxia-responsive genes strikingly upregulated in HIF-1α knock-down cells under chronic hypoxia

Transcript expression profiles of *IGFBP3, PAI1, LOXL2* and *EGFR* were determined in parental MCF-7 cells as well as in respective HIF-α knock-down derivatives. "C" indicates samples from cells cultured at ambient oxygen concentration while hypoxic ("hyp") probes were sampled from cultures exposed to 1% oxygen for 4, 8, 24, 48 and 72 hours, respectively. Basal expression levels as well as maximal hypoxic induction in parental cells are indicated with blue and red lines, respectively. Data are given as mean +SEM of three independent experiments and normalized to L28 mRNA levels. Highest expression levels in parental MCF-7 cells were arbitrarily defined 1 in each independet experiment.

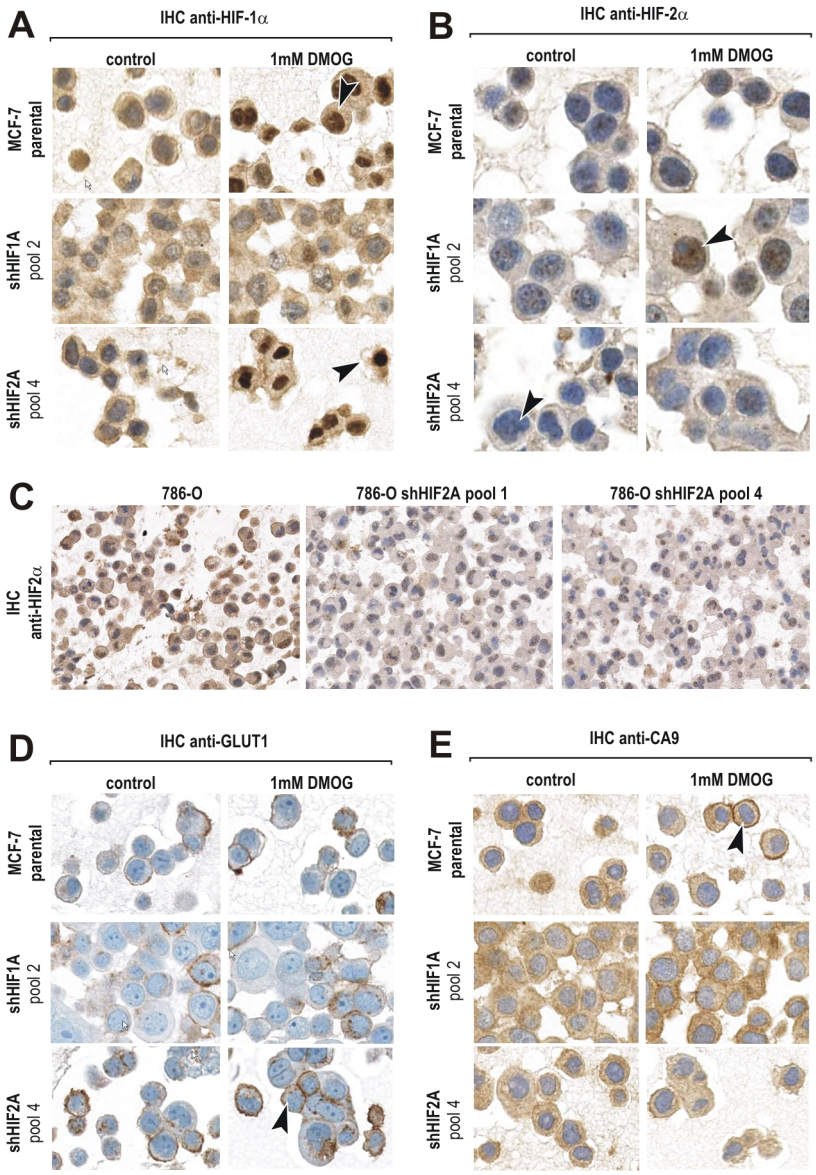
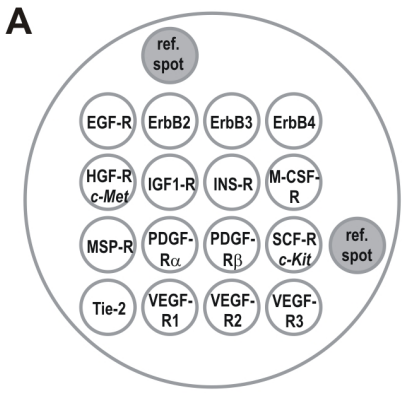


Figure S2. Validation of antibodies used for immunohistochemistry (IHC) on tissue microarrays (TMAs)

(A to E) Renal clear cell carcinoma 786-0 and breast cancer MCF-7 parental cells as well as respective cell pools with stable expression of shHIF1A and shHIF2A (1×10^8 cells) were cultured with or without 1mM DMOG for 24 hours and DMOG was readded 4 hours before harvest. Cell pellets were mixed with human plasma, coagulated by addition of thrombin and immediatly fixed by addition of 4% PFA solved in PBS. Coagulated pellets were embedded in paraffin and a cell pellet microarray was constructed as described before. Slides were subjected to an automated immunohistochemistry staining protocol using primary anti-HIF-1α, anti-HIF-2α, anti-GLUT1 and anti-CA9 with respective secondary antibodies. A strong nuclear signal for HIF-1α was observed only in DMOG treated MCF-7 parental and shHIF2A cell derivatives (see arrowheads) while shHIF1A pools were widely negative for HIF-1α, indicating specificity of this stain (A). HIF-2α staining showend a speckled nuclear pattern, which was most evident in DMOG treated shHIF1A pools (see

arrowhead). Note, that immunoblotting confirmed higher levels of HIF-2α in the absence of HIF-1α (see Figure 1). In nuclei of shHIF2A pools the speckled pattern was largely absent (B). Specificity of the HIF-2α signal was further confirmed by knock-down of HIF-2α in 786-0 cells which exclusively expresses HIF-2α. Due to deficiency of the von Hippel-Lindau protein (pVHL) in 786-0 cells, HIF-2α escapes the degradation machinery at oxic culturing conditions, resulting in nuclear and cytoplasmatic accumulation of HIF-2α



Symbol	*Name*	*Alias*
EGF-R	epidermal growth factor receptor	ErbB1/ErbB/HER-1
ErbB2	erythroblastosis oncogene B 2	HER-2/Neu
ErbB3	erythroblastosis oncogene B 3	HER-3
ErbB4	erythroblastosis oncogene B 4	HER-4
HGF-R	hepatocyte growth factor receptor	c-MET
IGF1-R	insulin-like Growth Factor 1receptor	CD221
INS-R	insulin receptor	CD220
M-CSF-R	macrophage colony-stimulating factor receptor	c-FMS/CSF1R / CD115
MSP-R	macrophage stimulating protein receptor	RON
PDGF-Ra	platelet derived growth factor receptor a	
PDGF-Rb	platelet derived growth factor receptor b	
SCF-R	stem cell factor receptor	c-KIT
Tie-2	tyrosine kinase with immunoglobulin-like and EGF-like domains 1	VMCM1/CD202b
VEGF-R1	vascular endothelial growth factor receptor 1	FLT1

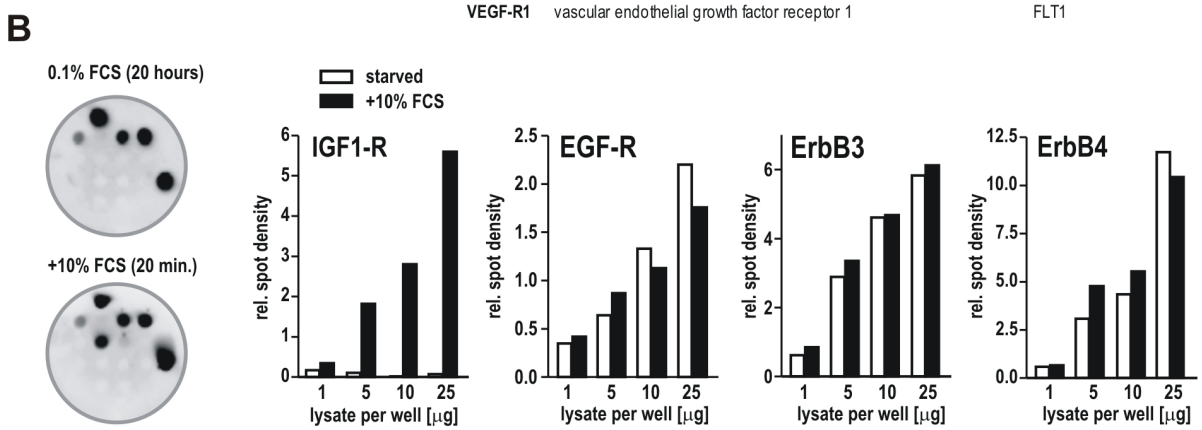


Figure S3. Autotrophic activation of EGF receptor family in MCF-7 breast carcinoma cells

(A) Human Phospho-RTK Proteome Profiler (R&D Systems) spot layout. Refer to the table for abbreviations and alternative gene product names ("ref. spot": reference spot). (B) IGF1-R is specifically activated in serum stimulated MCF-7 cells. Serum starved MCF-7 cultures (0.1% FCS for 20 hours) were stimulated with medium containing 10% FCS for 20 minutes and lysed according to the manufacturer's protocol. Receptor phosphorylation status of 16 RTKs was assessed by loading 1 to 25 □g of total protein per assay. A representative image of starved and stimulated MCF-7 cells is show (25 μg total protein). Phosphorylation levels of EGF receptor family and IGF1-R were quantified by measuring the spot intensities (Quantity One/Biorad).

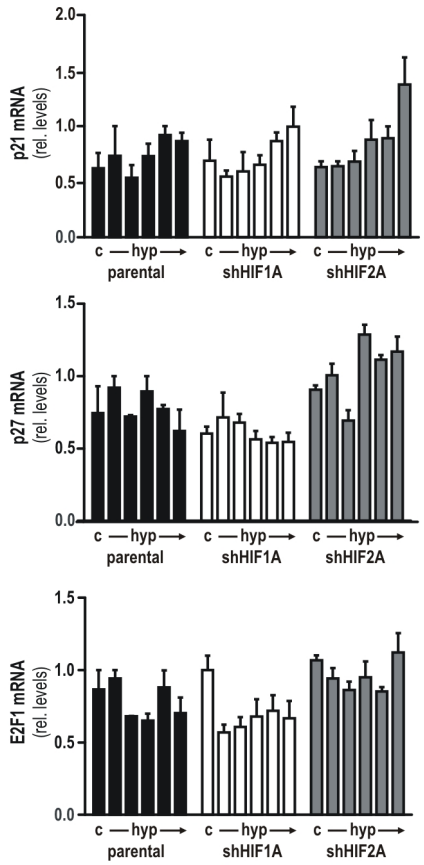


Figure S4. HIF-α knock-down only marginally affects c-*Myc* dependent gene expression in MCF-7 cells

Hypoxic transcript expression profiles of genes repressed by c-*Myc* activity (p21 and p27) as well as E2F1, which is activated by c-*Myc*, were determined in parental MCF-7 cells as well as in respective HIF-a knock-down derivatives. "C" indicates samples from cells cultured at ambient oxygen concentration while hypoxic ("hyp") probes were sampled from cultures exposed to 1% oxygen for 4, 8, 24, 48 and 72 hours, respectively. Data is given as mean +SEM of three independent experiments and normalized to L28 mRNA expression. Highest expression levels in parental MCF-7 cells were arbitrarily defined 1 in each independent experiment.

Table S1. Clinicopathological parameters of invasive breast cancer cases and relation to HIF-α isoform expression.

Variable	HIF-1α					HIF-2α				
	cases	*(%)*	low	high	*p*	cases	*(%)*	low	high	*p*
Age										
< 60 years	87	*(45.1)*	46	41	0.239§	84	*(46.9)*	53	31	0.049§
≥ 60 years	106	*(55.0)*	65	41		95	*(53.1)*	46	49	
Tumor histology										
invasive ductal	172	*(82.3)*	98	74	0.472#	159	*(82.0)*	91	68	0.191#
invasive lobular	28	*(13.4)*	17	11		26	*(13.4)*	9	17	
others	9	*(4.3)*	7	2		9	*(4.6)*	6	3	
Menopause										
pre	43	*(22.3)*	20	23	0.099§	42	*(23.5)*	28	14	0.091§
post	150	*(77.7)*	91	59		137	*(76.5)*	71	66	
Stage										
pT1	65	*(31.1)*	41	24	0.825§	60	*(30.9)*	25	35	0.140§
pT2	100	*(47.9)*	56	44		92	*(47.4)*	57	35	
pT3	10	*(4.8)*	3	7		10	*(5.2)*	5	5	
pT4	34	*(16.3)*	22	12		32	*(16.5)*	19	13	
pN0	73	*(40.8)*	53	20	0.001§	68	*(40.2)*	39	29	0.710§
pN1	87	*(48.6)*	43	44		83	*(49.1)*	45	38	
pN2	12	*(6.7)*	7	5		12	*(7.1)*	5	7	
pN3	7	*(3.9)*	1	6		6	*(3.6)*	4	2	
Grade										
1	39	*(18.7)*	26	13	0.109§	34	*(17.5)*	17	17	0.009§
2	108	*(51.7)*	63	45		101	*(52.1)*	46	55	
3	62	*(29.7)*	33	29		59	*(30.4)*	43	16	
Receptor status										
ESR-pos	161	*(83.4)*	97	64	0.136§	149	*(83.2)*	72	77	0.002§
ESR-neg	32	*(16.6)*	15	17		30	*(16.8)*	24	6	
PGR-pos	142	*(68.0)*	87	55	0.218§	131	*(67.9)*	61	70	0.001§
PGR-neg	67	*(32.1)*	35	32		62	*(32.1)*	45	17	
ErbB2 (0, 1+, 2+)	189	*(93.6)*	115	74	0.001§	177	*(93.2)*	95	82	0.278§
ErbB2 (3+)	13	*(6.4)*	1	12		13	*(6.8)*	9	4	

crosstables were analysed using §χ^2- tests for trends or #*Pearson's* χ^2- tests

Supplemental Experimental Procedures

mRNA quantification

cDNA was generated by reverse transcription (RT) of 1-3 µg of total RNA using AffinityScript reverse transcriptase (Agilent). Transcript levels were quantified by quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma) in combination with a MX3000P light cycler (Agilent). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. All data are expressed as ratios relative to ribosomal L28 mRNA levels. Melting point analyses of amplified PCR products were performed for each primer pair to verify specific amplification. Sequences of primers and length of specific amplicons (in base pairs) are given below.

Gene	Name	Accession	forward	reverse	amplicon
CA9	carbonic anhydrase IX	NM_001216	5'-gggtgtcatctggactgtgtt-3'	5'-cttctgtgctgccttctcatc-3'	309
PPFIA4	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4	NM_015053	5'-gaccacctcaccaagaagga-3'	5'-tgttgtggtcgaagttctcg-3'	292
PHD3	HIF prolyl hydroxylase 3	NM_022073	5'-atcgacaggctggtcctcta-3'	5'-cttggcatcccaattcttgt-3'	186
IGFBP3	insulin-like growth factor binding protein 3	NM_001013398	5'-acagccagcgctacaaagtt-3'	5'-aggctgcccatacttatcca-3'	269
ERO1L	ERO1-like (S. cerevisiae)	NM_014584	5'-tcaacagcgatttgatggaa-3'	5'-tgcagtcgaaagtcctcctt-3'	291
SFXN3	sideroflexin 3	NM_030971	5'-caggtcagaggcttggctac-3'	5'-tctcagctctggttccaggt-3'	278
LOXL2	lysyl oxidase like 2	NM_002318	5'-tgcttggaggacacagaatg-3'	5'-ctcttcgctgaaggaaccac-3'	297
ITRP1	inositol 1,4,5-triphosphate receptor, type 1	NM_001099952	5'-tctggctctgatcctcgttt-3'	5'-tctggctctgatcctcgttt-3'	222
HIF2A	hypoxia inducible factor 2 a	NM_001430	5'-ttgatgtggaaacggatgaa-3	5'-ggaacctgctcttgctgttc-3'	196
WISP2	WNT1 inducible signaling pathway protein 2	NM_003881	5'-ctcctctgcctcctctcaaa-3'	5'-gtctcccctttcccgatacag-3'	296
PAI1	plasminogen activator inhibitor type 1	NM_000602	5'-actggaaaggcaacatgacc-3'	5'-gaggaagggtctgtccatga-3'	296
PHD2	HIF prolyl hydroxylase 2	NM_022051	5'-gaaagccatggttgcttgtt-3'	5'-ttgccttctggaaaaattcg-3'	162
BNIP3	BCL2/adenovirus E1B 19 kda interacting protein 3	NM_004052	5'-aactgcacttcagcaataatgg-3'	5'-ccgacttgaccaatcccata-3'	311
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	NM_004566	5'-cttgtcgctgatcaaggtga-3'	5'-ttctgctcctccacgaactt-3'	243
HIF1A	hypoxia inducible factor 1 a	NM_001530	5'-tccgatggaagcactagaca-3'	5'-tggtgacaactgatcgaagg-3'	243
BHLHB3	basic helix-loop-helix domain containing, class B, 3	NM_030762	5'-gagcatgaaacgagacgaca-3	5'-gatcgctccccattctgtaa-3'	255
ANKZF1	ankyrin repeat and zinc finger domain containing 1	NM_018089	5'-gttccgaaggttcatggaga-3'	5'-ttgacgattgcagagtcagg-3'	303
E2IG5	Homo sapiens family with sequence similarity 162, member A	NM_014367	5'-cctacggattggcagaaaaa-3'	5'-tttggccttcatagctgctt-3'	270
VEGFA	vascular endothelia growth factor A	NM_001025366	5'-ctacctccaccatgccaagt-3'	5'-tggtgatgttggactcctca-3'	263
GLUT1	glucose transporter 1	NM_006516	5'-tcactgtgctcctggttctg-3'	5'-cctgtgctcctgagagatcc-3'	233
JMJD1A	jumonji domain containing 1A	NM_018433	5'-atggtgtctggagtgcatca-3'	5'-gtactcgggcagtggaatgt-3'	294
JMJD2B	jumonji domain containing 2B	NM_015015	5'-aacttcgacgatggctccta-3'	5'-tcctccagggtgaagatgtc-3'	233
YEATS2	YEATS domain containing 2	NM_018023	5'-cagaaacacctggaccgagt-3'	5'-cttggttttgaggggagtca-3'	335
ZNF395	zinc finger protein 395	NM_018660	5'-gtgcagctgaaggaggaatc-3	5'-ggtgagactgggatctggaa-3	290
MXI1	MAX interactor 1	NM_005962	5'-ctcaacaaagccaaagcaca-3'	5'-gcactggagtaaccctcgtc-3'	335
REDD1	protein regulated in development and DNA damage response 1	NM_019058	5'-cctggacagcagcaacagt-3'	5'-ccaggcgcagtagttctttg-3'	236
EGFR	epidermal growth factorreceptor (erythroblastic leukemiaviral (v-erb-b) oncogene homolog, avian)	NM_005228	5'-cagcgctaccttgtcattca-3'	5'-agctttgcagcccatttcta-3'	247
LOX	lysyl oxidase	NM_002317	5'-ccccaaagagtgaaaaacca-3'	5'-ccaggactcaatccctgtgt-3'	254
BHLHB2	basic helix-loop-helix domain containing, classB, 2	NM_00360	5'-gaccggattaacgagtgcat-3'	5'-gcttggccagatactgaagc-3'	283
PDK1	pyruvate dehydrogenase kinase, isozyme 1	NM_002610	5'-tcaccaggacagccaataca-3'	5'-atgggcaatccataaccaaa-3'	308
NDRG1	N-myc downstream regulated gene 1	NM_006096	5'-atgtacccctccatggatca-3'	5'-tgtggaccacttccacgtta-3'	298
ANGPTL4	angiopoietin-like 4	NM_139314	5'-agcatctgcaaagccagttt-3'	5'-gcgcctctgaattactgtcc-3'	278
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 4	NM_004567	5'-tgccaagagtctagcccagt-3'	5'-tgaccagcacattctcttgc-3'	323
ALDOC	aldolase C, fructose-bisphosphate	NM_005165	5'-gcctctagctgggactgatg-3'	5'-tgtacacagcagccaagacc-3'	302
p21	cyclin-dependent kinase inhibitor 1A	NM_000389	5'-gacaccactggagggtgact-3'	5'-ggcgtttggagtggtagaaa-3	299
p27	cyclin-dependent kinase inhibitor 1B	NM_004064	5'-ccggctaactctgaggacac-3'	5'-cttctgaggccaggcttctt-3'	221
E2F1	E2F transcription factor 1	NM_005225	5'-tgcagagcagatggttatgg-3	5'-atctgtggtgagggatgagg-3	265

Plasmids and reporter gene assays

The human *AREG* promoter plasmids pAREG(-641/+120), pAREG(-120/+16) and the human *WISP2* promoter have been described elsewhere (Fritah et al., 2006; Lee et al., 1999). A 5'-UTR regulatory element encompassing nucleotides +10 to +120 of human *AREG* was amplified by PCR and cloned into pGL3prom (Promega). Mutations of conserved WRE, CRE, E-Box and T-Box motifs have been generated by site-directed mutagenesis and are based on the minimal HIF-2α responsive AREG promoter construct pAREG(-120/+120) we recently described (Bordoli et al., 2010). Deletions of the 5'-UTR of pAREG(-120/+120) have been constructed by digestions with SmaI or SacI restriction enzymes and were designated accordingly. The PHD2 luciferase reporter plasmid pGL(P2P)607wt is a truncated version of the previously published human *PHD2* promoter (Metzen et al., 2005), while a 95 base pair element surrounding the HBS of the *PHD2* promoter was cloned into pGL3prom to obtain pSVL(P2P)95bp. Forced expression of HIF-2α in stable shHIF2A #4 knock-down cells was achieved using a hairpin-insensitive HIF-2α expression construct with silent mutations in the target region as indicated in the following primer (base exchanges underlined): 5'-GACCAATGC AGTACCCAAACTGACTTCAATGAGCTGGAC-3'. If not otherwise indicated, 1×10^6 MCF-7 cells or derivatives were transiently transfected with 200 ng reporter plasmid and 800 ng of either HIF-α or empty expression vectors in a six-well format by PEI transfection (Stiehl et al., 2006). To control for differences in transfection efficiency and extract preparation, 5 ng pRL-CMV *Renilla* luciferase reporter vector (Promega) were co-transfected. Cultures were evenly split onto 12-well plates 24 hours post transfection and exposed to 20% or 1% oxygen for an additional 24 hours. Luciferase activities of triplicate wells were determined using the Dual Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). Reporter activities were expressed as relative *Firefly/Renilla* luciferase activities.

Immunoblotting

Combined cytoplasmic and nuclear extracts were prepared using a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1× protease inhibitory cocktail (Sigma). Protein concentrations were determined by the

Bradford method and up to 200 µg cellular protein was subjected to immunoblot analyses. Membranes were probed with antibodies against HIF-1α (clone 54/HIF-1α, BD Transduction Laboratories), HIF-2α. (NB100-122, Novus Biologicals), PHD2 (NB100-137, Novus Biologicals), REDD1 (Proteintech Group) and β-actin (Sigma). Monoclonal antibodies against CA9 (M75) and PHD3 (P3-188e) were kind gifts of S. Pastorekova (Bratislava, Slovak Republic) and P. J. Ratcliffe (Oxford, UK), respectively. Signals from HRP-coupled secondary antibodies were detected with ECL substrate (all Pierce) using a luminescent image analyser (LAS-4000, FUJIFILM).

Cell proliferation/viability assays

Cells (2×10^4/well) were plated on 96-well plates and allowed to attach before cultures were grown at various serum concentrations (0-10% FCS) for an additional period of 72 hours at 20% or 1% oxygen. Relative viability of cultures was determined by cellular conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). In brief, cells were exposed to MTT (5 µg/ml) for 2 hours at 37°C and formazan crystals were solubilized with 50% dimethylsulfoxid, 25% dimethylformamide and 10% sodium dodecyl sulphate (pH 4.7). Absorbances at 570 nm were determined in a 96-well photometer and means of quadruplicates were calculated after background substraction. Data is expressed as relative values compared to cellular growth of each cell pool at 20% oxygen in the presence of 10% FCS.

Histopathologic grade and Immunohistochemistry

Construction of tissue microarrays (TMA) used for this study has been described earlier (Theurillat et al., 2007). Tumor histology was determined according to the criteria of the World Health Organization as defined in (Tavassoli and Devilee, 2003). Staging of the disease followed (Sobin and Wittekind, 2002), while tumors were graded according to the modified Bloom and Richardson system (Elston and Ellis, 1991). For statistical analyses, only cases with clinical follow-up were considered. TMA sections were processed using an automated immunohistochemistry (IHC) platform (Benchmark, Ventana, Roche, Illkirch, France). The follwing antibodies and dilutions were used: HIF-1α, clone mgc3 (abcam); HIF-2α, NB100-122 (Novus

Biologicals); ITPR1, HPA014765 (Sigma); AREG, HPA008720 (Sigma); PAI1, clone TJA6 (Leica/Novocastra); WISP2, LS-B917 (Lifespan Biosciences); CA9, ab10471 (Abcam); GLUT1, AB1340 (Chemicon); KRT5, clone D5/16B4 (Millipore); EGFR, clone 3C6 (Ventana); ErbB2, clone 10A7 (Leica/Novocastra); PGR, clone 1A6 (Millipore) and ESR, clone 6F11 (Ventana). IHC stainings for HIF-α, ESR and PGR were evaluated in percent of positive nuclei and expression levels were categorized as "low" (<median of overall expression) or "high" (>median of overall expression), respectively. Intensities of all other stainings were semiquantitatively scored as negative, weakly, moderate or strongly positive (0, 1+, 2+, 3+). Survival analyses stratified for AREG and WISP2 expression were performed considering negative and weak expression (0, 1+) as "low" and accordingly moderate and strongly positive (2+, 3+) as "high".

Bioinformatic analyses and statistical methods

Cluster analyses and visualization of gene expression data were performed using the GenePattern software package hosted on the Broad Institute website (http://www.broad.mit.edu/genepattern) (Reich et al., 2006). In brief, mean values of quantitative expression data derived from at least three independent hypoxic time courses were expressed on a $\log_2$ (ratio) scale with highest gene expression levels in parental MCF-7 arbitrarily defined as one. Elements were grouped into 6 clusters using consensus clustering to define the optimal number of clusters, and cluster centres were calculated applying the k-means algorithm. Samples were clustered using a hierarchical clustering algorithm and *Euclidean* distances of samples were visualized by dendrograms. In analogy, expression data from 50 cell lines were extracted from Kao *et al.* (Kao et al., 2009) and samples clustered using a hierarchical clustering algorithm. Conservation across species of promoter regions was evaluated using the MULTIZ whole-genome multiple alignment algorithm (Blanchette et al., 2004) implemented in the UCSC Genome Browser (http://genome.ucsc.edu). Alignments were manually edited using Genedoc version 2.6 (http://www.psc.edu/biomed/genedoc). For multiple comparisons, one-way ANOVA with *Tukey-Kramer's post hoc* test was performed. Column statistics applying unpaired *Student's* t-tests were calculated using GraphPad Prism version 4.0 (GraphPad Software). Protein expression data for statistical TMA evaluation were analysed in cross tables with χ^2 tests. Survival analyses were conducted according to *Kaplan-Meier* with log rank test and multivariate Cox regression analyses (inclusion model) using PASW (IBM SPSS Statistics 18) software. Area-proportional Convex

Venn-3 diagrams were drawn using *Peter Rodger's* (Canterbury, UK) web applet (Rodgers et al., 2010).

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7 Own contributions to publication and manuscript

MR Bordoli, DP Stiehl, L Borsig, G Kristiansen, S Hausladen, P Schraml, RH Wenger and G Camenisch, **Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis**, Oncogene. 2010 Sep. 20. [Epub ahead of print] doi: 10.1038/onc.2010.433

- Everything except shPHD2 single clone generation, shHIF-1α and shHIF-2α cell pools, figures 1B, 1D, and CRE site mutation in the AREG promoter.

DP Stiehl, **MR Bordoli**, I Abreu-Rodriguez, P Schraml, K Wollenick, G Kristiansen, RH Wenger, **HIF-2α dependent regulation of autonomous cell growth by the AREG-EGFR-ErbB4 axis is associated with significantly improved patient survival in human breast cancer** *(submitted)*

- Part of kinetics, mRNA and qRT-PCR leading to figures 1D, 2B and 2C. Figures 4A, 6A-B and supplementary Figures 1, 3. Supplementary Table 1.

8 Unpublished data

8.1 Loss of PHD2 in MCF-7 cells increases cell migration in vitro

8.1.1 Materials and methods

Human MCF-7 breast carcinoma cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma, Buchs, Switzerland) as described previously[216]. For hypoxia experiments, cells were grown in a gas-controlled glove box (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK).

The migration potential of MCF-7 cells was assessed in a boyden chamber transwell assay (8 μm pore filters, 24-well format). 600 μl FCS-containing medium was added to the outer chamber. Exponentially growing cells were washed with PBS and trypsinized. Next, cells were washed three times in FCS-free medium containing 1% BSA, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Washed cells were resuspended in FCS-free medium. 250'000 cells in a maximal volume of 300 μl were then seeded in the inner chamber. Plates were incubated 8 hrs in a normal incubator under normoxic or 1% O_2 conditions.

After incubation, medium was carefully aspirated and 750 μl of ice-cold MeOH added to the outer chamber. Plates were incubated 20 min at 4°C. Afterwards MeOH was aspirated and 0.5% crystal violet in 500 μl 20% MeOH added to the outer chamber. After 30 min staining at room temperature, crystal violet was washed away with water. The upper part of the filter was cleaned with a cotton swap to remove non-migrated cells. Migrated cells were quantified at the microscope.

For *in vivo* metastasis studies, cells were detached using PBS-EDTA and $5x10^5$ cells were injected intravenously into nude mice. After 5 weeks mice were sacrificed and the lungs perfused and excised for RNA extraction.

8.1.2 Results

To investigate the migratory behaviour of MCF-7 shPHD2 cells, we performed a boyden chamber migration assay. The two shPHD2 single clones C1 and C7 showed a significant increase in migration in response to FCS stimulus compared to wild type (wt) cells and cells transfected with the control shRNA. This increased migration rate could be observed under normoxic as well as under hypoxic conditions [Figures 1A and 1B]. Moreover, the PHD2 reconstituted clones #1, #2 and #5 showed again a decreased migration potential compared to the shPHD2 clone C1. The migration rate of the reconstituted clones did not differ from the one of wt or shcontrol cells [Figure 1C]. To exclude the possibility of a clonal artefact due to single clone selection, the experiment was repeated using a pool of lentivirally transfected shPHD2 cells. The increased migration rate of shPHD2 cells compared to the pool of cells transfected with control shRNA was confirmed [Figure 1D].
To investigate if the increased migration rate observed *in vitro* for shPHD2 cells also affects *in vivo* metastasis formation, MCF-7 parental, shPHD2 single clone C1 and the shPHD2 pool were injected intravenously into nude mice. After 5 weeks mice were terminated and organs excised. Despite the differences seen *in vitro,* no macroscopic metastasis could be observed in any of the animals. Measurements of human epithelial cell adhesion molecule (Epcam) mRNA levels in lung material did not show any difference between mice injected with parental cells and animals injected with shPHD2 cells [data not shown].

8.1.3 Discussion

We could show that loss of PHD2 in MCF-7 cells significantly increases their migration rate *in vitro* under normoxic as well as hypoxic conditions in response to an FCS stimulus. Reconstitution of PHD2 reduced the migration potential again to control levels. This enhanced migration might be due to the activation of the classical HIF pathway observed after down-regulation of PHD2. Many HIF target genes are known to regulate cancer relevant processes like cell migration, cell invasion and induction of metastasis. To support our hypothesis, the experiments

should be repeated using PHD2/HIF-1α and PHD2/HIF-2α double knock-down cell lines.

Intravenous injection of MCF-7 parental or shPHD2 cells into nude mice did not lead to any metastasis formation. From the literature, it is known that MCF-7 breast cancer cells are not invasive and only in rare cases induce metastasis. Our observations are therefore not surprising and can be explained with the very low metastatic potential of the MCF-7 cell line. To investigate the possibility that PHD2 knock-down cells are more metastatic , experiments should be carried out with a more invasive cell line.

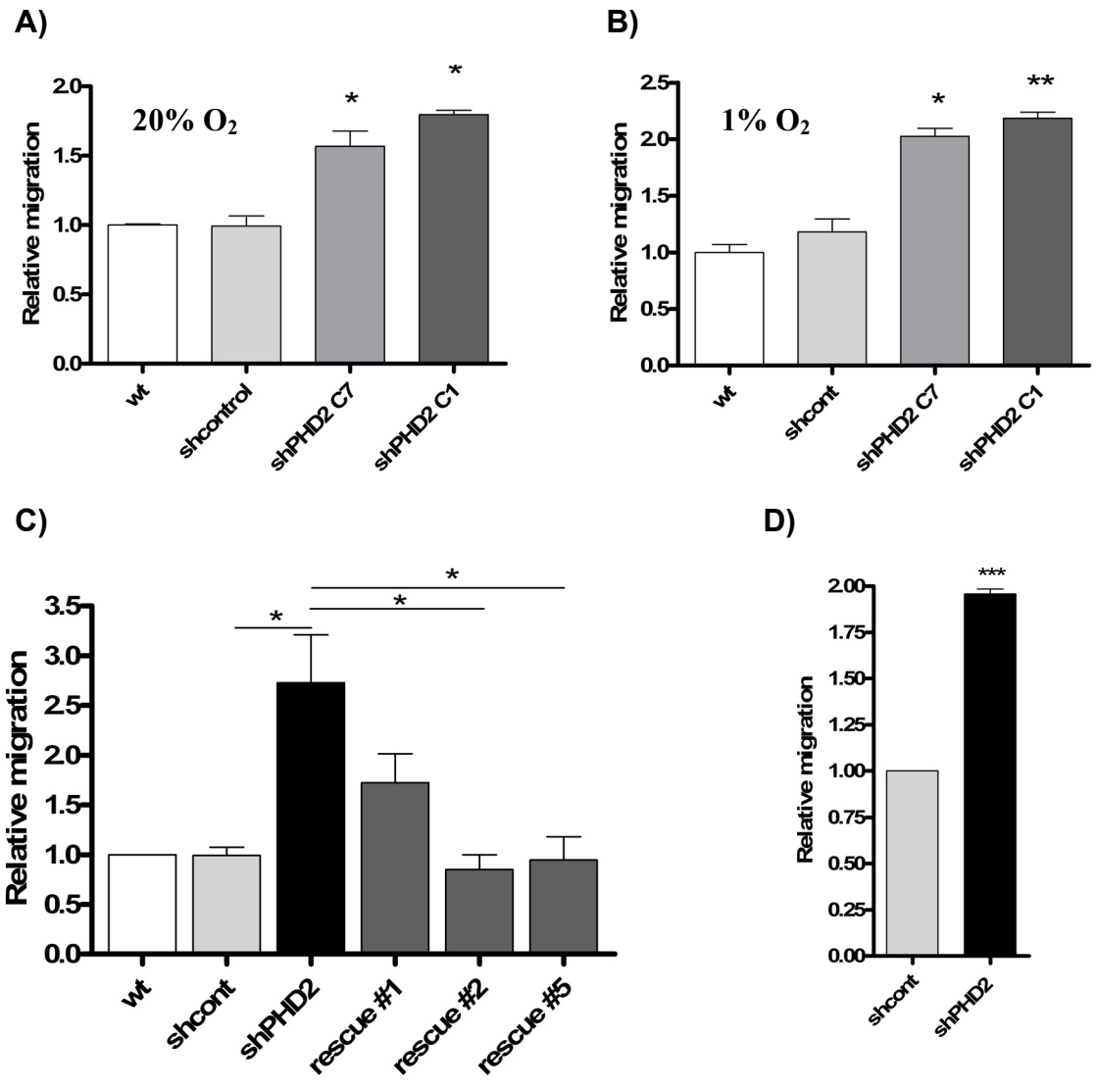

Figure 1. **PHD2 downregulation increases the migration rate of MCF-7 cells.** A) and B) ShPHD2 clones C1 and C7 showed a significantly increased migration compared to shcontrol transfected cells under normoxic (left) as well as hypoxic conditions (right). C) PHD2 reconstitution in the clones #1, #2 and #5 reduces the migration rate to control levels. D) Increased migration was observed in a pool of lentivirally transfected shPHD2 cells. Statistical analysis was performed using student t-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

8.2 Generation of stable PHD2 and PHD3 knock-down clones in MCF-7 and MDA-MB-231 breast cancer cells

8.2.1 Downregulation of PHD3 in MCF-7 cells

In addition to the previously described PHD2 knock-down clones in MCF-7 cells, we were also able to generate stable clones downregulating PHD3 in the same cell line. From the clone named #1.1 generated by Daniel Stiehl, an additional limited dilution with the aim to improve the downregulation of PHD3 was started. Selection of a large number of new single clones screened under normoxic as well as under dimethyloxaloylglycine (DMOG) treatment did not lead to improved PHD3 downregulation at the mRNA level. However, clones 3C4 and 3C6 showed reduced PHD3 levels compared to levels observed in the parental clone #1.1 [Figure 1A]. To further confirm efficiency and stability of the PHD3 downregulation, parental cells, clones #1.1, 3C4, 3C6 and #4.1 were incubated under hypoxic condition up to 72 hrs and PHD3 mRNA levels measured. Since clone #4.1 only showed a moderate PHD3 downregulation [data not shown] it was used as a control. Stable low PHD3 mRNA levels were confirmed in clones #1.1, 3C4 and 3C6, while late PHD3 induction was observed in the clone #4.1 [Figure 1B].

8.2.2 Double PHD2 and PHD3 downregulation in MCF-7 cells

Starting from stable shPHD2 single clones in MCF-7 cells, we aimed to generate stable double knock-downs for PHD2 and PHD3. Interestingly, although many cells survived the double antibiotic selection only 1 clone, named #5, showed an efficient down-regulation of both PHD isoforms [Figure 2] at the mRNA level. In all other selected cells, expression of either PHD2 or PHD3 was restored [data not shown] suggesting that concomitant downregulation of both PHD isoforms does not confer a selective advantage.

8.2.3 Downregulation of PHD3 in MDA-MB-231

As a second breast cancer cell model, we chose to work with the highly invasive and metastatic cell line MDA-MB-231. Interestingly, while MCF-7 is a hormone-dependent cell line expressing both the estrogen as well as the progesterone receptor, growth and proliferation of MDA-MB-231 is independent of presence of hormones. Therefore, the MDA-MB-231 is negative for both the previously mentioned receptors and offers an interesting alternative model to study the effects of PHDs downregulation in breast cancer.

The first aim was the generation of shPHD3 single clones. In contrast to MCF-7, where PHD3 mRNA levels are continuously increasing under hypoxia, in MDA-MB-231 PHD3 mRNA levels seem to peak after 8 hrs hypoxia and decrease again at later time points [Figure 3, open bars].

Efficient and stable PHD3 down-regulation at the mRNA level could be shown in clones C2, C4 and D2 [Figure 3].

8.2.4 Downregulation of PHD2 in MDA-MB-231

To generate MDA-MB-231 clones downregulating PHD2, we first tried the classical approach with polyethyleneimine (PEI)-mediated transfection of the shRNA construct, followed by limited dilution under selection pressure. Only few clones survived the selection. Out of them, clones B, E and F showed a moderate PHD2 downregulation at the mRNA level [Figure 4]. Interestingly, the proliferation rate of the shPHD2 clones, namely B and E, was strongly reduced compared to parental cells [Figure 5]. To exclude clonal artefacts coming from the limited dilution under strong selective pressure, the transfection of the shRNA for PHD2 was repeated using a lentiviral expression system. With this second attempt a pool of clones strongly and efficiently downregulating PHD2 could be generated. The efficiency of the downregulation was assessed via immunoblotting as shown in [Figure 6]. The effect on cell proliferation observed in the single clones could not be reproduced in this pool [data not shown].

8.2.5 Double PHD2 and PHD3 downregulation in MDA-MB-231

The generation of double knock-down of PHD2 and PHD3 starting from stably transfected shPHD3 MDA-MB-231 clones was unfortunately unsuccessful. Only a few single clones survived the limited dilution under double selection. Subsequent mRNA measurements clearly showed that only one PHD isoform was found to be convincingly downregulated, whereas the mRNA levels for the second isoform were comparable to parental cell levels. Therefore, the double selection led to clones strongly down-regulating either PHD2 (clones 3 and 4) or PHD3 (clones 1, 2 and 5) [Figure 7]. These results suggest that cells MDA-MB-231 lacking two out of the three PHD isoforms might not be viable or might have a very slow proliferation rate, which results in overgrowth by other cells.

A possible alternative approach would be a lentiviral double transfection and evaluation of a pool of clones instead of the time consuming and clonal selection prone limited dilution.

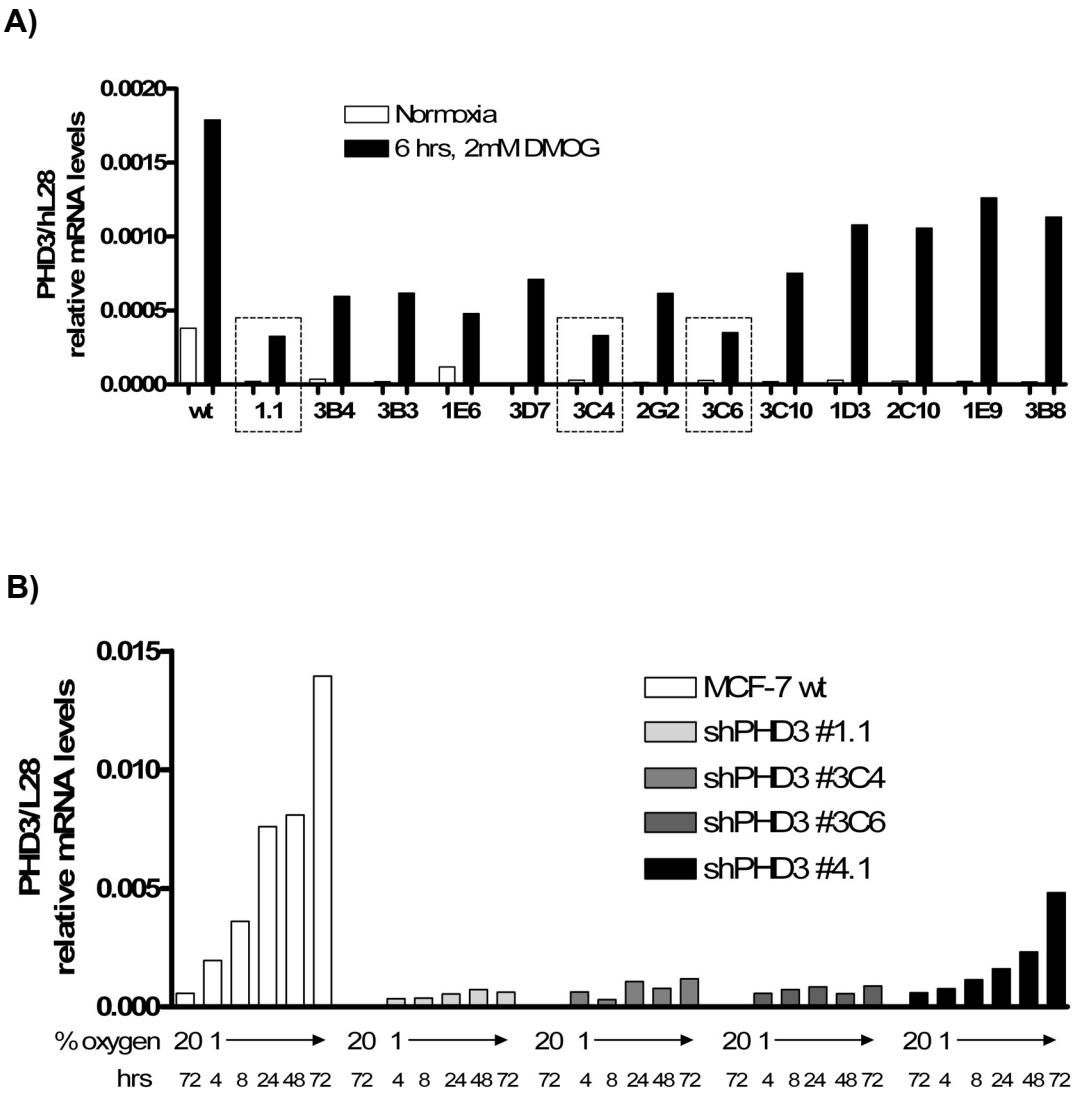

Figure 1. Stable and efficient downregulation of PHD3 in MCF-7 cells. A) Clone 1.1 was subjected to limited dilution; the new clones 3C4 and 3C6 showed a comparable down-regulation of PHD3 mRNA, measured by quantitative RT-PCR, in normoxia as well as under DMOG treatment. B) Hypoxic kinetics (1% O_2) of shPHD3 clones showing efficient and stable reduction of PHD3 mRNA levels.

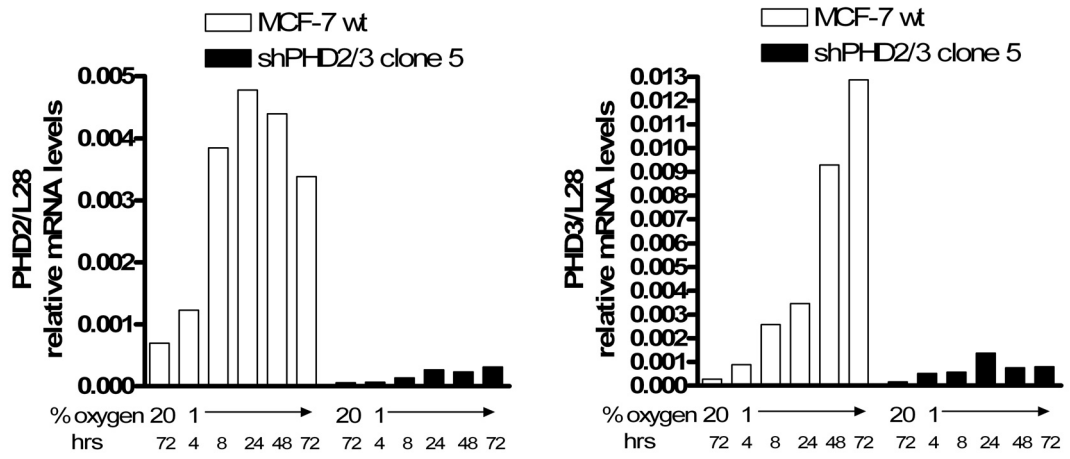

Figure 2. Stable and efficient concomitant downregulation of PHD2 and PHD3 in MCF-7 cells. mRNA levels of PHD2 (left panel) and PHD3 (right panel) were measured by quantitative RT-PCR. Both PHD2 and PHD3 mRNA levels were stably and efficiently reduced in clone 5 compared to parental cells.

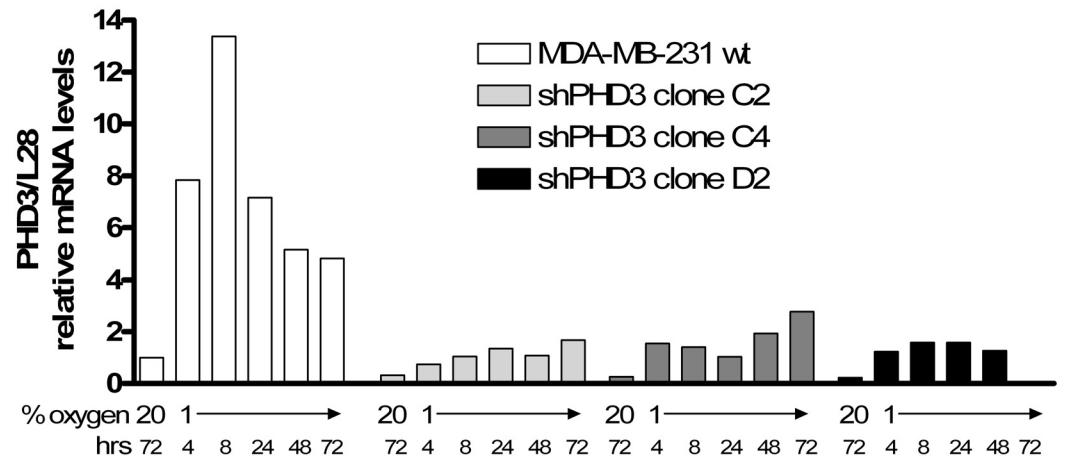

Figure 3. Stable and efficient downregulation of PHD3 in MDA-MB-231 cells. PHD3 mRNA levels were measured by quantitative RT-PCR**.** PHD3 levels were stably and efficiently reduced in the shPHD3 clones C2, C4 and D2.

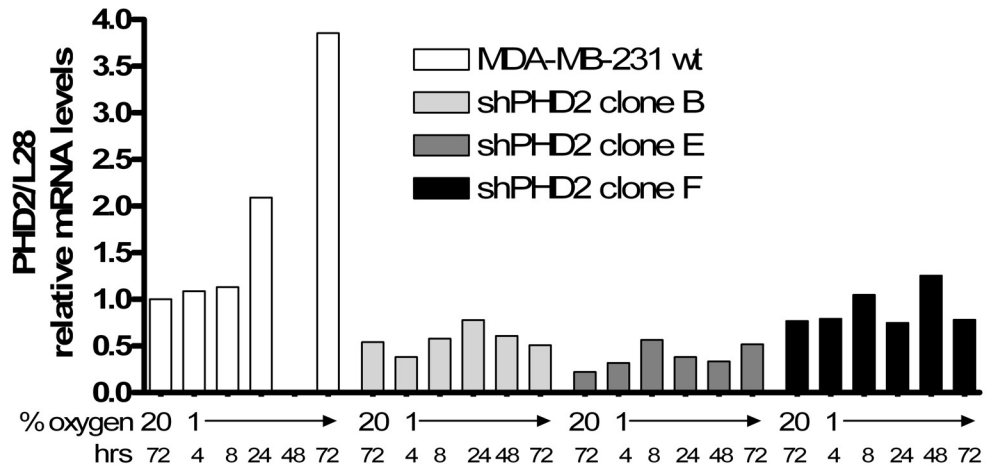

Figure 4. Stable and efficient downregulation of PHD2 in MDA-MB-231. PHD2 mRNA levels were measured by quantitative RT-PCR. The single clones B, E and F showed stable and efficient downregulation of PHD2 at the mRNA level.

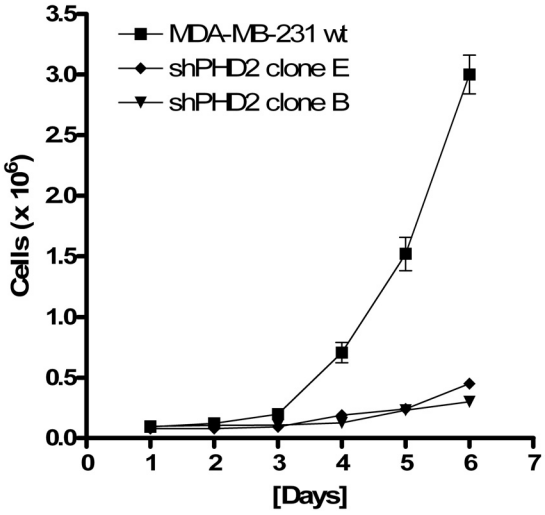

Figure 5. Reduced proliferation rate of MDA-MB-231 shPHD2 cells. Cells were counted every second day using a Beckman ViCell cell counter. shPHD2 clones B and E show a strong reduction in their proliferation rate compared to parental cells.

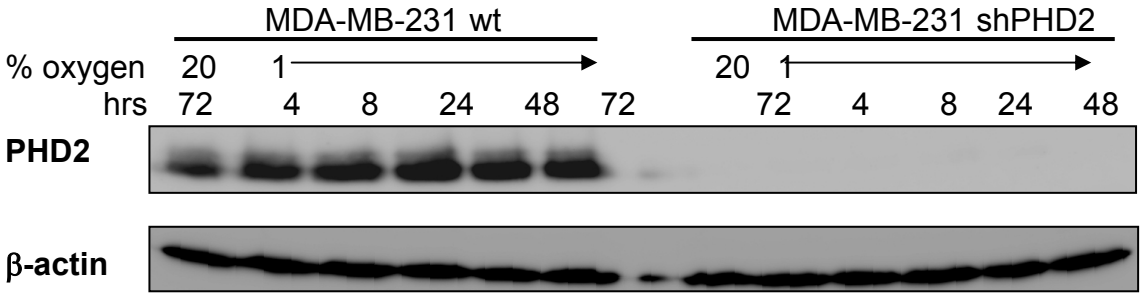

Figure 6. Stable and efficient lentivirally mediated downregulation of PHD2 in MDA-MB-231 cells. Protein was extracted from a pool of lentivirally transfected shPHD2 cells and PHD2 protein levels were compared to parental cells by immunoblotting. PHD2 was stably and efficiently down-regulated in the knock-down pool of clones also under prolonged hypoxia.

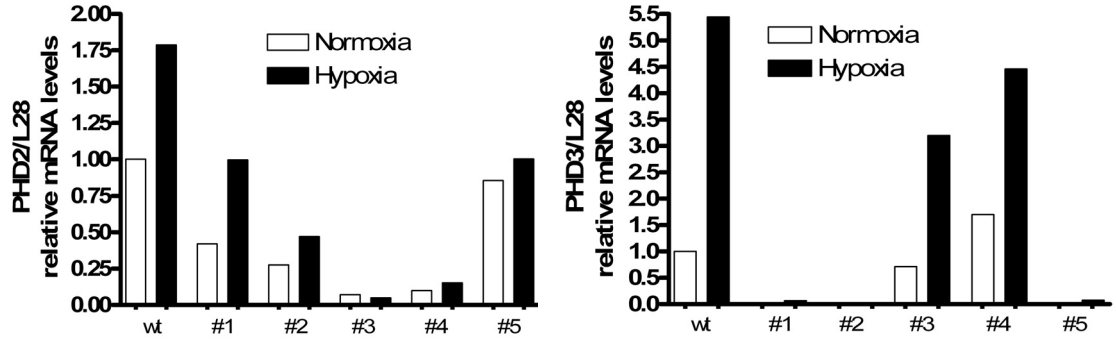

Figure 7. Simultaneous stable downregulation of PHD2 and PHD3 in MDA-MB-231 cells was not achieved. PHD2 (left panel) and PHD3 (right panel) mRNA levels were measured in different clones subjected to double transfection and double selection. Clones #3 and #4 showed reduced PHD2 levels, but normal PHD3 levels. Clones #1, #2, and #5 showed an almost complete PHD3 downregulation, however PHD2 levels were still high.

9 Discussion

9.1 Background

Reduced tissue oxygenation is a common feature of many pathological conditions like stroke, ischemia, inflammation and cancer. Most solid tumors are known to be at least partially hypoxic. The degree of tumor hypoxia is an important adverse prognostic marker.

The most important players in regulating short-term answers as well as adaptation to hypoxia are the hypoxia-inducible transcription factors. The HIF-α subunit is hydroxylated in the presence of oxygen by PHD proteins. Hydroxylation at two specific proline residues, located within the ODD domain, targets HIF-α for degradation. Under hypoxic conditions, absence of oxygen inhibits PHD activity, causing HIF-α stabilization and its translocation to nucleus. Nuclear dimerization of the HIF-α subunit with the constitutively expressed β subunit and subsequent DNA binding at specific HREs triggers the transcription of HIF target genes. Many well known HIF target genes are involved in the malignant progression of cancer. Among them the chemokine receptor CXCR4 was shown to play an important role in determining the metastatic destination of breast cancer cells[217], lysyl oxidase (LOX) that was shown to be critical for the metastatic niche formation after its secretion from hypoxic breast tumors[218, 219] and the extensively studied VEGF-A, a key player in angiogenesis.

To investigate the role of PHD2 in the context of breast cancer, we generated stable PHD2 knock-down clones in MCF-7 cells. Xenografts experiments in nude mice showed a clear correlation between PHD2 levels and tumor size. Immunohistochemical analysis of the excised tumors showed a significant higher vascularization in the absence of PHD2. Secretion of angiogenic factors like VEGF, IL-8 and AREG was found to be modulated by PHD2. Specific HIF-2α and PHD2-dependent AREG regulation could be confirmed at the mRNA level as well as on the promoter level. Our results suggest a role for PHD2 as a tumor suppressor in breast cancer.

9.2 Role of PHD2 and AREG in breast tumorigenesis

AREG represented an interesting target, since it was first discovered in conditioned media of MCF-7 cells[220]and was recently shown to play an important role in breast cancer initiation and progression[221, 222]. AREG overexpression is often found in breast tumors especially in invasive mammary carcinomas[223, 224] and was shown to induce cell invasion in MCF-10A cells[225]. Even more important in the context of our work was the fact that AREG antisense nucleotides have been used to reverse the malignant phenotype of a transformed human breast epithelial cell line *in vivo*[226]. The observed reduction of tumor mass correlated with a reduced tumor vascularization. Also the fact that AREG is a heparin-binding glycoprotein belonging to the epidermal growth factor (EGF) family, which binds the epidermal growth factor receptor 1 (EGFR)[220], makes it an interesting candidate. In fact, EGF signaling is one of the most important pathways involved in the development of breast cancer as well as in the malignant progression of mammary carcinomas. Although we did not specifically investigate if the phenotypic differences in tumorigenesis observed in our breast cancer model were entirely due to PHD2-dependent regulation of AREG by generation and injection into mice of PHD2/AREG double knock-down clones, we could at least provide evidence for the importance of AREG in angiogenesis by *in vitro* tube formation assays. In addition, our mouse experiments and our clinical data suggest a role for PHD2 as a tumor suppressor in breast cancer.

9.3 Role of PHD2 and AREG in angiogenesis

Interestingly, the most striking differences seen in *in vitro* tube formation assays concerned the extent and the size of the branching points that were altered after downregulation of AREG. This observation is in line with the known developmental role of AREG. In fact, AREG was shown to play an essential role during ductal morphogenesis in the pubertal mammary gland[227] and to induce epithelial branching in organ cultures of embryonic mouse kidneys[228]. Taken together, our results and previous studies suggest a specific role for AREG in stimulating the morphogenesis of branching substructures in different organs and blood vessels. Since AREG is for sure not the most potent angiogenic factor known, it was somehow surprising to

observe such a difference in *in vitro* tube formation. Notably, the clear reduction of branching points after addition of the anti-AREG blocking antibody occurred in the presence of relatively high levels of VEGF and IL-8, which were also found to be increased after downregulation of PHD2. Mechanistically, very little is known on how AREG performs its angiogenic function. It has been shown that AREG down-regulation reduced the expression levels of transforming growth factor β 1 (TGFβ1) in a transformed breast epithelial cell model[229]. A further study proposed a link between AREG and tumor necrosis factor α (TNFα) mediated IL-8 upregulation in human airway epithelial cells[230]. We did not investigate, if our described IL-8 upregulation in the absence of PHD2 was a direct consequence of PHD2 downregulation, similarly to previously published data[214], or a secondary effect due to the induction of AREG.

9.4 Role of oxygen in AREG regulation

Since the PHD2 enzymatic function is strongly dependent on pO_2 and the main PHD2 hydroxylation target, HIF, as well as NF-kB, recently described as a PHD2 target[214], are strongly influenced by oxygen availability, we analyzed the hypoxic inducibility of AREG. We could show that AREG mRNA as well as AREG promoter activity were not induced under hypoxic conditions (1% O_2). However, it remains to be analyzed whether AREG is transcriptionally regulated under lower oxygen conditions or anoxia. Of note, AREG mRNA levels were unchanged after DMOG treatment, a hypoxia mimicking condition. In the literature the effects of oxygen on AREG are still unclear. In experiments performed with premature rat lungs as a model of BPD, AREG was shown to be induced after hyperoxia[231]. Microarray studies with colon-derived epithelial cells showed an increase in AREG after hypoxic exposure (1% O_2). This hypoxic upregulation of AREG could be confirmed by RT-qPCR. Further experiments excluded an involvement of the HIF pathway, but showed that the hypoxic AREG induction was depending on cyclic AMP response element binding protein (CREB)[232].

In line with the lack of hypoxic induction, we could show that PHD2-mediated regulation of AREG promoter activity was not dependent on PHD2 hydroxylation

activity. Based on our experimental results, we conclude that AREG transcription in MCF-7 breast cancer cells is not affected by oxygen levels.

9.5 Transcriptional regulation of AREG

Mechanistically, we concentrated our efforts on investigating, if the HIF pathway was involved in the PHD2-dependent expression of AREG. Activation of the HIF pathway is well known to lead to increased angiogenesis through the activation of HIF target genes like VEGF. AREG mRNA levels were found to be strongly reduced after HIF-2α downregulation in MCF-7 cells and weakly induced after HIF-1α downregulation. In addition, we could induce AREG promoter activity in a HIF-2α knock-down background by exogenous HIF-2α expression, whereas overexpression of HIF-1α did not show any effect. We hypothesized that the weak increase in mRNA levels observed after HIF-1α down-regulation was probably due to the higher HIF-2α levels under these particular conditions. The HIF-2α mediated regulation of AREG has not been reported before. AREG transcription has been shown to be induced by the Wilms Tumor suppressor (WT) 1 transcription factor during development in a model of fetal kidney differentiation[228]. Moreover, estrogen was also shown to be implicated in transcriptional induction of AREG, probably indirectly, since no estrogen-responsive elements (ERE) were identified in the AREG promoter[233]. As mentioned before, CREB was previously found to be important for hypoxic AREG induction[232] and we could confirm by mutation of the CRE binding site in the AREG promoter that CREB affects AREG promoter activity. However, the PHD2 effect on AREG promoter activity was maintained despite the mutation of the CRE site. Furthermore, REL-A downregulation in PHD2 knock-down cells did not alter mRNA expression of AREG, suggesting that in contrast to the published PHD2/NF-κB/IL-8[214] or ANG axis, PHD2-mediated regulation of AREG does not occur via NF-κB signaling. Recently, BRCA1, another fundamental protein involved in breast cancer development and progression, has been proposed as a transcriptional repressor of AREG further underlying the importance of this gene in the molecular context of mammary carcinoma[234].

Moreover, we tried to identify which region of the AREG promoter is essential to mediate the HIF-2α effect. Whereas deletion of a major part of the AREG 5'-

untranslated region (UTR) abolished the HIF-2α-dependent AREG induction, transfection of the isolated 5'-UTR failed to confer HIF-2α responsiveness to a reporter construct driven by the SV40 minimal promoter. These results suggest that the AREG 5'-UTR is necessary but not sufficient to mediate the HIF-2α effect and that additional sites might be involved. Conserved sites in the AREG promoter, including WT1, CREB, E-box and T-box response elements, were identified by phylogenetic foot-print analyses. Only mutation of the WT1 response element (WRE) significantly attenuated the specific responsiveness to HIF-2α, whereas mutation of the other sites did not influence the HIF-2α effect on the promoter. Although WT1 was identified as a potent transcriptional activator of the WRE site[228], we can exclude its involvement since we could not detect any WT1 mRNA expression in our cells (data not shown). Absence of WT1 expression in MCF-7 cells was already described in a previous study[235]. Therefore we conclude that AREG expression in response to HIF-2α might at least in part be due to an undefined WRE binding activity involving direct or indirect HIF-2α binding or binding of other HIF-2α-induced transcription factors.

9.6 The HIF-2α/AREG/EGFR axis and autonomous cell growth

Analysis of the phosphorylation status of 16 receptor tyrosine kinases (RTKs) using an RTK array, clearly showed that only members of the EGFR family are active in MCF-7 cells, namely EGFR, ERBB3 and ERBB4. Furthermore, the phosphorylation status of EGFR and ERBB4 was found to be reduced in the absence of HIF-2α when compared to parental cells. In the absence of HIF-1α, the phosphorylation status of the mentioned receptors was found to be increased, possibly due to higher HIF-2α activity in these cells. Importantly, phosphorylation of EGFR and ERBB4 in HIF-2α lacking cells could be restored by addition of exogenous AREG. These results underline the importance of EGFR signaling in MCF-7 cells and suggest a role for HIF-2α in mediating the activation of the EGFR pathway. Although reduced EGFR signaling after HIF-2α depletion was recently observed[236], to our knowledge, AREG provides the first direct link between HIF-2α dependent transcription and EGFR family activation in human breast cancer. Moreover, when exposed to low

oxygen concentration, HIF-2α depleted cells showed a dramatic retardation in proliferation compared to parental cells. Additionally shHIF-2α cells were also found to be highly susceptible to serum depletion. Hypoxic conditions associated to nutrient deprivation are a common feature of solid tumors and the impaired proliferation observed in HIF-2α depleted cells suggest a role for HIF-2α in regulating autonomous growth factor signaling. Taken together our data led to the conclusion that cells expressing HIF-2α benefit from autocrine and paracrine growth factor signaling, which supports cellular autonomy, decreasing the selection pressure and possibly slowing tumor progression. Supporting this hypothesis, our clinical data showed a significant increased overall survival of patients with high HIF-2α tumors. Similar observations were recently made in a non-small cell lung cancer model, where HIF-2α depletion resulted in an increase of tumor burden[237].

9.7 Potential clinical application of PHD inhibitors

Our results as well as other recent studies identified PHD2 as a potential tumor suppressor protein. This hypothesis was corroborated by clinical data from patients suffering from breast or colon carcinoma as well as from *in vivo* xenografts experiments using various PHD2 down-regulated cell lines[214]. Since PHD inhibitors have been developed as EPO substitutes as well as for the treatment of ischemic diseases, potential adverse effects on tumor progression should be taken into account.

In this context however, not only the role of PHD2 in tumor cells, but also the role of stromal PHD2 should be considered. In fact, in addition to our results and to the already mentioned studies also the influence of “host” PHD2 on tumor growth was examined. Using a genetically modified mouse model heterozygous for PHD2, it was shown that orthotopic tumors grown into wild-type mice were more invasive and prone to generate metastasis compared to tumors grown in a *Phd2*$^{+/-}$ genetic background[215]. Although no difference could be detected in overall tumor vessel density, PHD2 was shown to dramatically influence the morphology of the blood vessels. The reduced intravasation of tumor cells into the circulatory system of *Phd2*$^{+/-}$ mice and the consequent reduction of metastatic spread was due to more regular, smoother and mature blood vessel compared to the malformed and leaky

vessels found in tumors derived from wild-type mice[215]. Based on these observations, one can hypothesize a beneficial effect of PHD2 inhibition in the stroma leading to vessel normalization in the tumor and preventing generation of distant metastasis.

Additional studies addressing simultaneously both the effects of silencing PHD2 in the host and/or silencing PHD2 in the tumor cells would be of interest to further elucidate the complex role of this enzyme in cancer. Furthermore, clinical administration of PHD inhibitors for the treatment of non-cancerous diseases should be carefully evaluated. Parameters like predisposition to cancer, presence of tumors, tumor type as well as systemic or local administration of the drugs should be considered. Since application of pan-PHD inhibitors in rats stimulated angiogenesis[81], clinical trials combining PHD inhibitors together with anti-angiogenic compounds might be of interest.

10 References

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11 Curriculum Vitae

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Publication list

MR Bordoli, DP Stiehl, L Borsig, G Kristiansen, S Hausladen, P Schraml, RH Wenger and G Camenisch, **Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis**, Oncogene. 2010 Sep. 20. [Epub ahead of print] doi: 10.1038/onc.2010.433

K Balamurugan, V-D Luu, MR Kaufmann, VS Hofmann, G Boysen, S Barth, **MR Bordoli**, DP Stiehl, H Moch, P Schraml, RH Wenger and G Camenisch, **Onconeuronal cerebellar degeneration-related antigen, Cdr2, is strongly expressed in papillary renal cell carcinoma and leads to attenuated hypoxic response,** Oncogene 2009; 28 (37): 3274-85

DP Stiehl, **MR Bordoli**, I Abreu-Rodriguez, P Schraml, K Wollenick, G Kristiansen, RH Wenger, **HIF-2α dependent regulation of autonomous cell growth by the AREG-EGFR-ErbB4 axis is associated with significantly improved patient survival in human breast cancer** *(submitted)*

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